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13. ABSTRACT (Maximum 200 Words) The original proposed specific tasks of this Idea grant were threefold: (1) to identify the cis-acting elements and the trans-acting factors that are responsible for the cytokine oncostatin M (OM)-induced suppression of p53 transcription in breast cancer cells, (2) to determine whether ERK and STAT3 play essential roles in OM-mediated suppression of p53 transcription, and (3) to over express p53 in a tetracycline regulated expression system. These 3 tasks have been successfully accomplished. We have identified a novel cis-regulatory element, designated as PE21, that mediates the OM-induced suppression of p53 transcription. By conducting electrophoretic mobility shift assay connected with UV-crosslinking, we have detected a protein of 87 kDa that specifically binds to the PE21 element. We have demonstrated that blocking STAT3 transactivating activity by the expression of a dominant negative mutant of STAT3 (dnStat3) reversed the OM inhibitory effects on p53 promoter activity and p53 protein expression, indicating an involvement of STAT3 in OM-mediated negative regulation of the p53 transcription. In addition, to determine functional roles p53 in the process of proliferation and differentiation of breast cancer cells, we generated stable cell lines (MCF-7 ptsp53) that express p53Val ¹³⁵ temperature-sensitive mutant. We found that overexpression of functional p53 in MCF-7 cells leads to growth arrest at the G ₂ /M phase of the cell cycle without an induction of apoptosis.				
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INTRODUCTION

Oncostatin M (OM), a 28 kDa glycoprotein, is a cytokine produced by activated T lymphocytes and macrophages (1). Our previous studies showed that OM inhibits the growth of several breast cancer cell lines, including MCF-7, MDA-MB231, and H3922, which is a cell line derived from an infiltrating ductal carcinoma (2-6). Breast cancer cells respond to OM treatment with reduced growth rates and the appearance of differentiated phenotypes. However, OM treatment does not appear to lead to apoptosis. Since the p53 tumor suppressor protein plays important roles in cellular proliferation and differentiation, we examined the effects of OM on p53 expression in breast cancer cells. Surprisingly, we found that p53 expression was down regulated by OM in MCF-7, MDA-MB231, and H3922 cells (7). Decreased levels of p53 protein and mRNA were detected after 1 day of OM treatment and reached maximal suppression of 10-20% of control after 3 days. Nuclear run-on assays further demonstrated that OM decreased the number of actively transcribed p53 mRNA. In order to delineate the molecular mechanisms by which OM regulates p53 transcription and to understand the signaling transduction pathways that are involved in the OM-mediated transcription of p53 we have conducted a series of experiments using a variety of molecular approaches and biochemical methods to identify the cis-acting elements and the trans-acting factors that are responsible for the OM-induced suppression of p53 transcription. In addition, we have utilized MAP kinase inhibitors and the dominant negative mutants of STAT3 and STAT1 to delineate the signaling transduction pathways involved in p53 regulation. Finally, we have generated stable cell lines (MCF-7 ptsp53) that express p53Val¹³⁵ temperature-sensitive mutant and utilize this cell line to identify p53 regulated genes.

BODY

The expression of p53 tumor suppressor gene in breast cancer cells is down-regulated by cytokine oncostatin M (appendix No. 1)

Previously, we showed that oncostatin M (OM), a cytokine produced by activated T cells and macrophages, inhibited the proliferation of breast cancer cells derived from solid tumors and malignant effusions. OM-treated cells showed reduced growth rates and differentiated phenotypes. Since the p53 tumor suppressor protein plays an important role in cellular proliferation, we examined p53 protein expression in three OM-responsive breast cancer cell lines, MCF-7, MDA-MB231, and H3922. Western blot analysis showed that p53 protein levels in all three cell lines were decreased by OM treatment. Reduction of p53 protein was detected after 1 day of OM treatment and reached maximal suppression of 10-20% of control after 3 days in H3922, and 40% of control after 4 days in MCF-7 cells. A comparison of p53 mRNA in OM-treated cells versus untreated control cells showed that exposure to OM reduced the steady-state levels of p53 mRNA transcripts to a similar extent as the p53 protein levels. This observation suggests that the effect of OM on p53 protein expression does not occur at the post-translational level. Nuclear run-on assays verified that OM decreased the number of actively transcribed p53 mRNAs, suggesting a transcriptional regulatory mechanism. The effect of OM on p53 expression appears to be mediated through the extracellular signal-regulated kinase (ERK) pathway, as inhibition of ERK activation with a specific inhibitor (PD98059) to the ERK upstream kinase MEK abrogated the OM inhibitory activity on p53 expression in a dose dependent manner. In addition to OM, we showed that the p53 protein expression in MCF-7 cells was also decreased by PMA treatment. Since both OM and PMA induce MCF-7 cells to differentiate, our data suggest that p53 expression in breast cancer cells is down-regulated during the differentiation process.

Identification of the novel OM-responsive regulatory element of the p53 promoter (Appendix No. 2)

Previously we showed that oncostatin M (OM) suppresses p53 gene transcription in breast cancer cells. To identify the cis-acting regulatory elements that mediate the OM effect, in this report, we dissected the p53 promoter region and analyzed the p53 promoter activity in MCF-7 cells. We show that treatment of MCF-7 cells with OM induced a dose- and time-dependent suppression of p53 promoter activity. The p53 promoter activity was decreased to 35% of control at 24 h and further decreased to 20% at 48 h by OM at concentrations of 5 ng/ml and higher. Deletion of the 5'-flanking region of the p53 promoter from -426 to -97 did not affect the OM effect. However, further deletion to -40 completely lost the OM-mediated suppression. The region -96 to -41 contains the NF- κ B and c-myc binding sites, and a newly identified UV-inducible element PE21. Mutations to disrupt NF- κ B binding or c-myc binding to the p53 promoter decreased the basal promoter activity without affecting the OM-mediated suppression, whereas mutation at the PE21 motif totally abolished the OM effect. We further demonstrate that insertion of PE21 element upstream of the thymidine kinase minimal promoter generated an OM response analogous to that of the p53 promoter. Finally, we detected the specific binding of a nuclear protein with a molecular mass of 87 kDa to the PE21 motif. Taken together, we demonstrate that OM inhibits the

transcription of p53 gene through the PE21 element. Thus, PE21 element is functionally involved in p53 transcription regulated by UV-induction and OM suppression.

STAT3 participates in OM-mediated downregulation of p53 (Appendix No.3)

OM downregulates p53 expression in MCF-7 cells by inhibiting the gene transcription (1,4). Since blocking the MEK/ERK pathway only partially reversed the OM inhibitory effect on p53 protein expression, it is possible that other signaling pathways could also be involved. OM activates both STAT3 and STAT1 in MCF-7 cells. To determine whether STAT3 or STAT1 activation is a key event in the OM-induced growth inhibition of MCF-7 cells, we established stable MCF-7 clones that express a dominant negative STAT3 mutant (dnStat3, Y705F). MCF-7 clones (neo) transfected with the empty vector (pEFneo) were also generated and were used in this study as negative controls to access possible side effects associated with antibiotic selection.

We examined p53 protein levels in MCF-7 neo and dnStat3 clones untreated or treated with OM. Western blot analysis shows that while OM treatment lowered p53 protein level to 35% of control in the neo clone, the level of p53 protein in the dnStat3 clone was not decreased by OM treatment. Taken together, these results demonstrate that activation of STAT3 signaling pathway is a necessary step in the OM-mediated regulation of p53 transcription.

Molecular characterization of oncostatin M-induced growth arrest of MCF-7 cells expressing a temperature-sensitive mutant of p53 (Appendix No.4)

Our previous studies have shown that treatment of MCF-7 breast cancer cells with cytokine oncostatin M (OM) results in a growth arrest and a concurrent decrease in p53 expression. It remains to be determined whether these two important events are directly connected, as changes in p53 protein levels can lead to variable biological outcomes. In this study we have generated stable cell lines (MCF7-ptsp53) that express p53Val¹³⁵ a p53 temperature-sensitive mutant. We demonstrate that overexpression of the wildtype (wt) p53 at permissive temperature in MCF7-ptsp53 cells leads to growth arrest at the G₂-M phase of the cell cycle. Inhibition of endogenous p53 function with the expression of mutant p53 protein at non-permissive temperature did not affect the OM-induced G₁ cell cycle arrest. Microarray studies were further carried out to identify p53- and OM-regulated genes that mediate the G₂/M or G₁ cell cycle arrest. We show that the expression of p21 was upregulated and expressions of cdc2, cyclin B2 and protein regulator of cytokinesis 1 (PRC1) were suppressed by overexpression of the wt p53 in MCF7-ptsp53 cells at the permissive temperature. In contrast, OM treatment caused coordinate changes of mRNA expression of several cell cycle components including c/EBP δ , cdc20, and thymidine kinase 1 (TK1) that mainly affect G₁-S phase transition. All together, our results suggest that the downregulation of p53 transcription may be involved in some other cellular changes induced by OM but it is not directly connected to the antiproliferative activity of OM *per se*.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of a novel Sp1 binding site that contributes to the maximal p53 basal transcriptional activity in breast cancer cells.
- Demonstration of the critical functional role of the newly identified regulatory element PE21 motif in OM-induced transcriptional suppression of the p53 gene.
- Providing the first evidence that an 87 kDa DNA binding protein interacts with the PE21 sequence.
- Establishment of a stable cell line (MCF-7 ptsp53) that can turn on or turn off the wild-type p53 expression by temperature switch.
- Through these studies we have identified the Protein Regulator of Cytokinesis (PRC1) as a novel p53 regulated gene and demonstrated that wt p53 strongly inhibits PRC1 gene transcription. The effects of wt p53 on the PRC1 gene expression are also confirmed in other cancer cells by transfection of wt p53 into T47D and HeLa cells. PRC1 has been shown to play an exclusive role in cytokinesis. In the absence of PRC1 expression, cells progress through mitosis normally but are unable to divide, leading to the formation of binucleate cells. In accordance with the decreased PRC1 expression, we have found that about 10% of MCF7-ptsp53 cells became binucleate after switching cells from 37°C to 32°C. Thus, identification of PRC1 as the p53 targeted gene may uncover a previously unrecognized function of p53 in the regulation of cytokinesis.

REPORTABLE OUTCOMES

- Two manuscripts have been published in the first 18 months of the award period that demonstrated the effects of OM on p53 transcription (Cell growth and Differentiation. 10:677-683, 1999) and identified a novel regulatory element PE21 on p53 promoter (*Oncogene* 20:8193-8202, 2001).
- Two manuscripts have been published in the third year of funding that characterized the OM-signaling pathway involved in p53 transcriptional regulation (*Oncogene* 22:804-905, 2003) and the identification of p53 regulated genes (*Breast Cancer Res. Treat.*, In press).

CONCLUSIONS

Transcriptional regulation of the p53 gene contributes to the change in expression of wildtype p53 during the cell cycle and to the elevated expression of mutated p53 in tumor cells. However, currently, little is known regarding the regulation of p53 transcription in tumor cells. Characterization of the human p53 promoter to localize the OM responsive elements to PE21 element provides new information for understanding the transcriptional control of p53 expression in breast cancer cells. Our studies of cell cycle and microarray analyses suggest that OM and p53 arrest cell growth by affecting different phases of the cell cycle; OM arrests cells at the G₀/G₁ phase whereas p53 halts the cycle progression at the G₂/M phase. In addition to regulate the cell growth, OM has been shown to induce many changes in cellular functions such as cell morphology, adhesion, motility, metabolism, and extracellular matrix deposition. Similarly, p53 controls numerous critical cellular processes. It has been shown that changes in p53 levels lead to variable outcomes. Given the consideration of the importance of p53 in cell homeostasis, the regulation of OM on p53 transcription warrants further investigation. Moreover, our novel finding of the regulatory effect of p53 on PRC1 gene expression is important. The activities of p53 on G1 and G2 checkpoints have been well studied. However, it has never been reported that p53 has a function in cytokinesis. Further investigations are needed to understand the functional significance of p53 regulation of PRC1 gene expression.

REFERENCES

1. Zarling, J.M., Shoyab, M., Marquardt, H., Hanson, M.B., Lionbin, M.N., and Todaro, G.J. Oncostatin M: a growth regulator produced by differentiated lymphoma cells. *Proc.Natl.Acad.Sci.USA.*, 83: 9739-9743, 1986.
2. Horn, D., Fitzpatrick, W.C., Gompper, P.T., Ochs, V., Bolton-Hanson, M., Zarling, J.M., Malik, N., Todaro, G.J., and Linsley, P.S. Regulation of cell growth by recombinant oncostatin M. *Growth Factors*, 2: 157-165, 1990.
3. Liu, J., Spence, M.J., Wallace, P.M., Forcier, K., Hellstrom, I., and Vestal, R.E. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth and down-regulation of the c-myc proto-oncogene. *Cell Growth.&Differ.*, 8: 667-676, 1997.
4. Spence, M.J., Vestal, R.E., and Liu, J. Oncostatin M-mediated transcriptional suppression of the c-myc gene in breast cancer cells. *Cancer Research*, 57: 2223-2228, 1997.
5. Douglas, A.M., Goss, G.A., Sutherland, R.L., Hilton, D.J., Berndt, M.C., Nicola, N.A., and Begley, C.G. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. *Oncogene*, 14: 661-669, 1997.
6. Douglas, A.M., Grant, S.L., Goss, G.A., Clouston, D.R., Sutherland, R.L., and Begley, C.G. Oncostatin M induces the differentiation of breast cancer cells. *Int.J.Cancer*, 75: 64-73, 1998.
7. Liu, J., Li, C., Ahlborn, T.E., Spence, M.J., Meng, L., and Boxer, L.M. The expression of p53 tumor suppressor gene in breast cancer cells is down-regulated by cytokine oncostatin M. *Cell Growth.&Differ.*, 5: 15-18, 1999.

The Expression of *p53* Tumor Suppressor Gene in Breast Cancer Cells Is Down-Regulated by Cytokine Oncostatin M¹

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Abstract

Previously (J. Liu, *et al.*, *Cell Growth Differ.*, 8: 667-676, 1997), we showed that oncostatin M (OM), a cytokine produced by activated T cells and macrophages, inhibited the proliferation of breast cancer cells derived from solid tumors and malignant effusions. OM-treated cells showed reduced growth rates and differentiated phenotypes. Because the *p53* tumor suppressor protein plays an important role in cellular proliferation, we examined *p53* protein expression in three OM-responsive breast cancer cell lines, MCF-7, MDA-MB231, and H3922. Western blot analysis showed that *p53* protein levels in all three of the cell lines were decreased by OM treatment. Reduction of *p53* protein was detected after 1 day of OM treatment and reached maximal suppression of 10-20% of control after 3 days in H3922 and 40% of control after 4 days in MCF-7 cells. A comparison of *p53* mRNA in OM-treated cells versus untreated control cells showed that exposure to OM reduced the steady-state levels of *p53* mRNA transcripts to an extent similar to that of the *p53* protein levels. This observation suggests that the effect of OM on *p53* protein expression does not occur at the posttranslational level. Nuclear run-on assays verified that OM decreased the number of actively transcribed *p53* mRNAs, which suggests a transcriptional regulatory mechanism. The effect of OM on *p53* expression seems to be mediated through the extracellular signal-regulated kinase (ERK) pathway, inasmuch as the inhibition of ERK activation with a

specific inhibitor (PD98059) to the ERK upstream kinase mitogen/extracellular-regulated protein kinase kinase abrogated the OM inhibitory activity on *p53* expression in a dose-dependent manner. In addition to OM, we showed that the *p53* protein expression in MCF-7 cells was also decreased by phorbol 12-myristate 13-acetate treatment (PMA). Because both OM and PMA induce MCF-7 cells to differentiate, our data suggest that *p53* expression in breast cancer cells is down-regulated during the differentiation process:

Introduction

OM,³ a *M_r* 28,000 glycoprotein, is a cytokine derived from activated T lymphocytes and macrophages (1-3). OM is a member of the IL-6 family cytokines, which includes IL-6, IL-11, LIF, ciliary neurotrophic factor, and cardiotrophin-1 (4-7).

As a pleiotrophic cytokine, OM elicits many different biological functions in different cell types, among which its ability to regulate cell growth and differentiation is most notable. OM stimulates the growth of normal fibroblasts (8, 9), normal rabbit vascular smooth muscle cells (10), myeloma cells (11), and AIDS-related Kaposi's sarcoma cells (12). OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including breast carcinoma, melanoma, and lung carcinoma (8, 9, 13-16). The inhibitory or stimulatory effects of OM on cell growth seem to depend on target cell type.

The growth regulatory activity of OM has been examined in a number of breast cancer cell lines, including MCF-7, MDA-MB231, and H3922 (8, 13-16). The common responses of breast cancer cells to OM are reduced growth rates and the appearance of differentiative phenotypes. The growth-inhibitory effects of OM in these cell lines were accompanied by striking morphological changes (13, 15). Similar to the morphological changes seen in H3922 cells (13), the appearance of cytoplasmic vacuoles and enlargement of cytoplasm were observed in MCF-7 and MDA-MB231 cells. In addition, OM-treated MDA-MB231 cells became spindle shaped, and the intercellular junctions were severely disrupted. In MCF-7 cells, a large amount of lipid droplets appeared after OM treatment. These phenotypic changes have been described as signs of differentiation of breast cancer cells (17). The

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³ The abbreviations used are: OM, oncostatin M; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; LIF, leukemia-inhibitory factor; MAP, mitogen-activated protein; MEK, mitogen/extracellular-regulated protein kinase kinase; OSMR β , oncostatin M-specific receptor β subunit; PMA, phorbol 12-myristate 13-acetate; LDL, low-density lipoprotein.

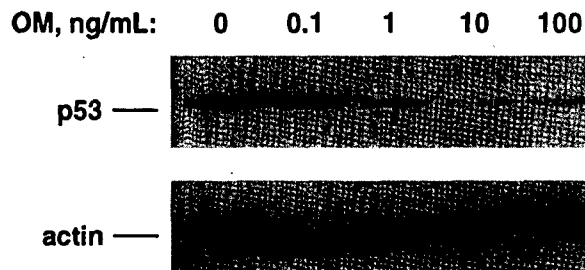


Fig. 1. Dose-dependent suppression of p53 protein expression by OM. Total cell lysate was isolated from H3922 cells that were untreated or treated with 0.1, 1, 10, or 100 ng/ml OM for 3 days. Untreated control cells and OM-treated cells were lysed simultaneously at the end of treatment. Fifty μ g of total cell lysate per sample were analyzed for p53 protein expression by Western blot. Anti- β -actin monoclonal antibody was used to normalize the amount of cellular protein being used. The normalized p53 protein levels expressed as % of control were determined by densitometric analysis of the immunoblot. The normalized p53 protein levels (% of control) are: control, 100; OM 0.1 ng/ml, 83; OM 1 ng/ml, 68; OM 10 ng/ml, 9; OM 100 ng/ml, 15. The figure shown is representative of two separate experiments.

examination of several breast cancer cell lines that were treated with OM did not show a significant number of apoptotic cells, which suggests that OM does not induce apoptosis in breast cancer cells. The expression of the proto-oncogene *c-myc* is induced by OM within 1 h and is subsequently suppressed by OM after 24–48 h (13, 14). The molecular and cellular mechanisms underlying the growth-inhibitory activity of OM have not been elucidated.

Because the p53 tumor suppressor protein plays important roles in cellular proliferation and transformation (18), we investigated the possibility that OM-induced growth inhibition and induction of differentiation are associated with alterations in p53 protein expression. In this study, we show that the p53 protein and the p53 mRNA in breast cancer cells were down-regulated by OM. Nuclear run-on studies and an analysis of p53 promoter activity both suggested that OM suppressed the transcription of the p53 gene. Interestingly, the effect of OM on p53 expression seemed to be mediated at least in part through the MAP kinase, also known as the ERK pathway, because the inhibition of ERK activation partially abolished the inhibitory effect of OM on p53 expression.

Results

To investigate whether OM regulates p53 expression, we initially examined the effect of OM on H3922 breast cancer cells. H3922 is a breast cancer cell line derived from an infiltrating ductal carcinoma. We characterized this cell line with regards to OM receptor, LIF receptor, EGF receptor, and estrogen receptor expression status (13). H3922 cells express highly abundant OM-specific receptors and respond to OM with a strong growth inhibition. Treatment of these cells with OM for 3 days reduced DNA synthesis to 20% of control at concentrations of 10–20 ng/ml (13, 14). H3922 cells were exposed to OM at different concentrations for 3 days, and then untreated and OM-treated cells were lysed. Total cell lysate was harvested, and 50 μ g of soluble protein from each sample were loaded on a 10% SDS gel and separated by electrophoresis, transferred to PVDF membrane, and blotted

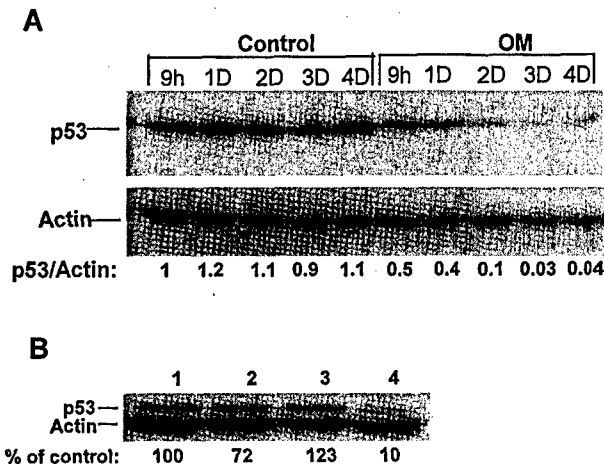


Fig. 2. Time course of regulation of p53 protein expression by OM. A, H3922 cells were untreated or treated with 50 ng/ml OM for 9 h, 1, 2, 3, and 4 days respectively. At the indicated times, cells—untreated or treated with OM—were lysed simultaneously. Twenty μ g of protein of total cell lysate per sample was analyzed for p53 and β -actin protein expression by Western blot. The figure shown is representative of two separate experiments. B, cells were seeded in regular growth medium overnight and then switched to serum-free medium for 48 h to induce growth arrest. Cell proliferation was initiated by adding serum (10% FBS) back to the culture medium with or without OM for 24 h. At the end of the experiment, all of the cells were harvested simultaneously and analyzed for p53 and β -actin expression by Western blot. Lane 1, cells cultured in regular growth medium for 48 h; Lane 2, cells cultured in serum-free medium for 48 h; Lane 3, after serum starvation, cells were incubated in regular growth medium for 24 h in the absence of OM; Lane 4, after serum starvation, cells were incubated in regular growth medium for 24 h in the presence of OM.

with anti-p53 monoclonal antibody. Fig. 1 shows that the p53 protein level was decreased in OM-treated cells in an OM dose-dependent manner. A maximal effect of 80% suppression was observed at 10 ng/ml and higher. In contrast to p53, the level of β -actin was not altered by OM.

We next examined the time course of OM regulation on p53 protein expression. H3922 cells were untreated or treated with 50 ng/ml OM for different period of times. The results showed that the levels of p53 protein in untreated control cells remained unchanged during the experiment but were markedly decreased by OM treatment. The amount of p53 in OM-treated cells decreased to 50% of control after 9 h, lowered to 40% after 24 h, and further declined to the level of 10% of control after 48 h (Fig. 2A). To establish that the effect of OM on down-regulation of p53 expression is not the result of growth arrest, H3922 cells were cultured in serum-free medium for 48 h, which led to growth arrest as assayed by [3 H]thymidine incorporation (data not shown). Then cell proliferation was initiated by replacing the culture medium with IMDM containing 10% FBS in the absence or the presence of OM for 24 h. Fig. 2B shows that the p53 protein level in serum-starved cells decreased 30% (Lane 2) as compared with that in cells cultured in regular growth medium (Lane 1). Adding serum back to the growth arrested cells stimulated p53 expression (compare Lane 3 with Lane 2) in the absence of OM. However, OM produced a similar inhibitory effect on p53 protein expression as seen in Fig. 2A. These experiments suggest that OM has a direct effect on

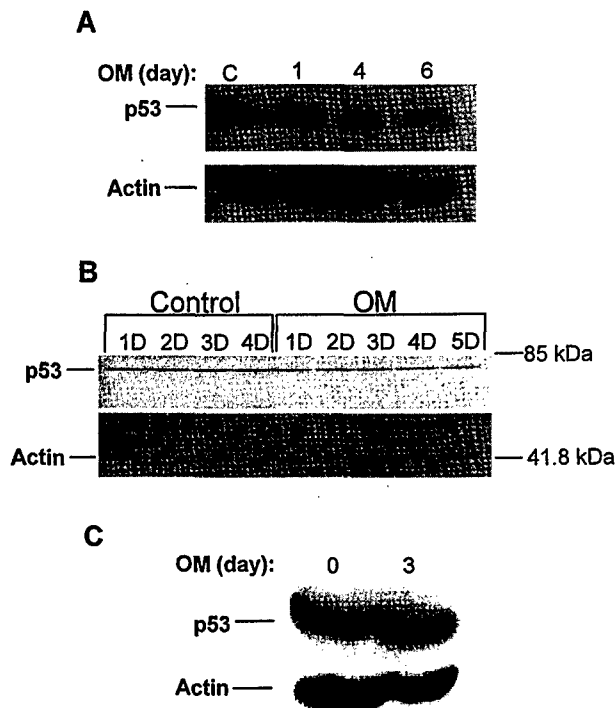


Fig. 3. Examination of regulation of p53 protein expression by OM in MCF-7, MDA-MB231, and H3477 cells. Cells cultured in 2% FBS IMDM were treated with 50 ng/ml OM for the indicated times, and the total cell lysate was isolated and analyzed for p53 protein by Western blot. A, total cell lysate of samples from MCF-7; B, total cell lysate of samples from MDA-MB231; C, total cell lysate of samples from H3477. The relative p53 protein levels were determined by densitometric analysis of the immunoblot and normalized to the signal of β -actin.

p53 expression independent of the growth status of the cells. However, these studies cannot rule out completely the possibility that the growth arrest induced by OM contributes partially to the decreased expression of p53.

To determine whether the inhibitory effect of OM on p53 is limited to the H3922 cell line or is a common response of breast cancer cells to OM, the effect of OM on p53 protein expression was examined in two other OM-inhibited breast cancer cell lines, MCF-7 and MDA-MB231. As shown in Fig. 3A, the p53 protein level in MCF-7 cells was decreased to 84% of control after 1 day of OM treatment, reaching a lowest level of 33% of control after 6 days. The parallel experiment of counting the cell number showed that treating MCF-7 cells with OM for 6 days reduced the number of viable cells to 42.3% of control. Comparing the treated cells with untreated cells, we did not observe a significant increase in the number of dying cells in OM-treated MCF-7 cells. Similar to MCF-7 and H3922 cells, the p53 protein expression in MDA-MB231 cells was also down-regulated by OM. In comparison with the control cells, the level of p53 protein in OM-treated cells decreased 30% after 1 day and 56% after 2 days (Fig. 3B). These data demonstrate that decreased p53 protein expression is associated with the growth inhibition exerted by OM on breast cancer cells.

To ensure that the observed effect of OM on p53 protein expression is a receptor-mediated event, we examined the

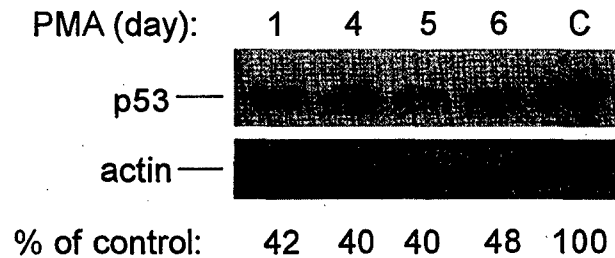


Fig. 4. Down-regulation of p53 protein expression by PMA. MCF-7 cells were treated with 100 ng/ml PMA for the indicated times and the p53 protein was detected by Western blot. The normalized p53 protein levels against actin were expressed as % of control and were determined by densitometric analysis of the immunoblot.

regulation of OM on p53 in H3477 cells. H3477 cells have been shown not to express the OM-specific receptor, and OM does not inhibit the growth of these cells (13, 19). As we expected, the p53 protein expression in H3477 breast cancer cells was not suppressed by OM (Fig. 3C), which indicated that the suppression of p53 protein expression by OM is a receptor-mediated event.

Previously (17, 20), it had been reported that PMA induces MCF-7 cells to differentiate. This differentiation process was accompanied by the accumulations of lipid droplets and intracellular vacuoles. To investigate the possibility that PMA down-regulates p53 expression in MCF-7 cells, MCF-7 cells were treated with 100 ng/ml PMA for different time periods, and p53 protein expression was examined by Western blot. Fig. 4 showed that the incubation of MCF-7 cells with PMA for 1 day lowered the steady level of p53 protein to 42% of that seen in control cells, and the p53 protein level remained at that low level at longer time points. Compared with the kinetics of OM action on p53 protein expression, the inhibitory effect of PMA seemed to be faster than OM in MCF-7 cells. Similarly, the PMA-induced morphological changes of MCF-7 cells were observed after 1 day of treatment, but the OM-induced morphological changes were not apparent until after 3–4 days (data not shown). Nevertheless, the common effects of OM and PMA on cell differentiation and p53 expression suggest that p53 expression in breast cancer cells is down-regulated during the process of cell differentiation.

Because the regulation of p53 protein expression has been shown to occur at levels of translation and transcription, the effect of OM on p53 mRNA expression was examined by Northern blot analysis. H3922 cells were treated with OM for different periods of times, and total RNA was isolated. Fig. 5 shows that the level of p53 mRNA, after normalization to GAPDH, was decreased to 60% of control after 9 h of OM treatment, was further lowered to 25% of control in cells treated with OM for 24 h, and the mRNA level remained constant at longer time points. These results indicate that the effects of OM on p53 mRNA and p53 protein expression are essentially correlated with regard to the kinetics and the degree of suppression.

To further study the regulatory mechanism, nuclear run-on assays were conducted to measure the relative rate of transcription of p53. Nuclei were prepared from control H3922 cells and H3922 cells treated with OM for 24 h, and tran-

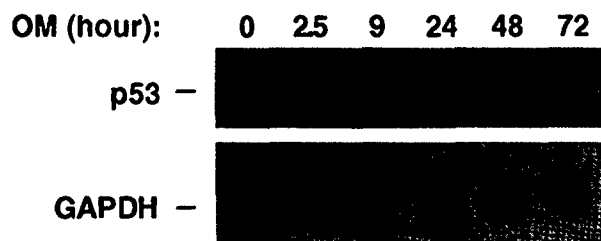


Fig. 5. Kinetics of *p53* mRNA expression in H3922 cells treated with OM. H3922 cells cultured in IMDM containing 2% FBS were incubated with 50 ng/ml OM for different times as indicated. Untreated control cells and OM-treated cells were lysed simultaneously at the end of the treatment with the RNA isolation solution. Total RNA was isolated and 15 μ g per sample was analyzed for *p53* mRNA by Northern blot. The membrane was stripped and hybridized to a human *GAPDH* probe. The figure shown is a representative of three different experiments.

scription was allowed to continue in the presence of [32 P]-UTP for 30 min. The incorporation of 32 P into *p53*-specific RNA was used as a measure of transcription rate. The transcription rate for *GAPDH* was also measured as an internal control. OM-treated cells contained only approximately 20% as many active *p53* transcripts as observed in control cells (Fig. 6A). Data were normalized by the signals detected in *GAPDH* slots. The effect of OM on *p53* transcription was further studied by analyzing *p53* promoter activity. A human *p53* promoter reporter construct, pGL3-*p53*LUC, and a human LDL receptor promoter reporter construct, pLDLR234LUC, were transiently transfected into H3922 cells, and the luciferase activities were measured. As shown in Fig. 6B, OM treatment decreased *p53* promoter activity to approximately 30% of control. In contrast, in the same experiment, the promoter activity of the LDL receptor was increased 2.5-fold, which is consistent with the stimulatory effect of OM on this promoter reported in other cell lines (21). These results obtained from the studies of nuclear run-on and promoter activity suggest that transcriptional regulation is a major component of the observed OM-mediated suppression of *p53* mRNA expression.

It has been proposed that the *p53* gene is a target of the proto-oncogene *c-myc*. The c-Myc protein regulates the transcription of *p53* through the c-Myc-responsive element present in the promoter region of the *p53* gene (22). We investigated the possibility that OM-mediated suppression of *p53* transcription was due to an effect on c-Myc-mediated transcription. Northern blot analysis was used to examine the levels of *c-myc* mRNA and *p53* mRNA in total RNAs isolated from H3922 cells untreated or treated with OM for different periods of time. Fig. 7 shows that transcription of the *c-myc* mRNA and the *p53* mRNA were not concurrently regulated by OM. The *c-myc* mRNA was increased more than 3-fold by OM after 30 min and slowly decreased afterward. The suppression of *c-myc* transcription was not seen until 24 h after OM treatment. In contrast, the *p53* mRNA level was steadily decreased during OM treatment. The biphasic effect of OM on *c-myc* mRNA was not seen. A similar result was obtained from MDA-MB231 cells. Furthermore, Western blot to examine the c-Myc protein level in untreated and OM-treated

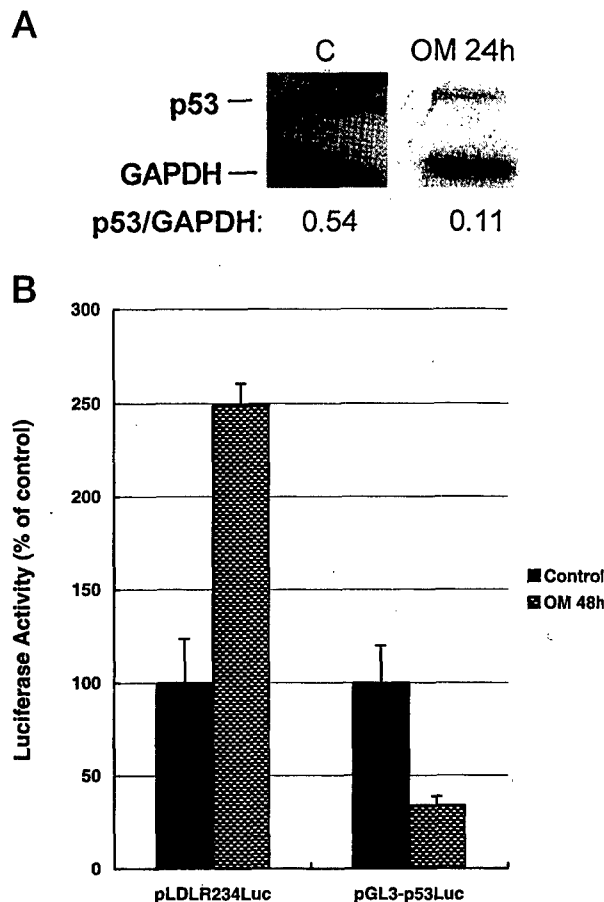


Fig. 6. OM down-regulates *p53* gene transcription. A, nuclear run-on analysis of *p53* transcription: two slots were blotted onto each of two nylon membrane strips. One slot received 3 μ g of the 2-kb fragment of the *p53* cDNA. The second slot was loaded with 5 μ g of the *GAPDH* plasmid. One nylon strip was hybridized to a 32 P-radiolabeled nuclear run-on reaction prepared from 24-h OM-treated H3922 cells. The second was hybridized to a labeled nuclear run-on reaction prepared from untreated control cells. Equal amounts of radioactivity were used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis. The figure shown is representative of two different experiments. B, analysis of human *p53* promoter activity: H3922 cells were transfected with the pGL3-*p53*LUC and pLDLR234LUC. After transfection, cells were cultured in media minus or plus OM for 48 h. Assays of luciferase activity were conducted as described (21). The data (mean \pm SE) shown are representative of three separate experiments in which triplicate wells were assayed.

H3922 cells showed that the level of c-Myc protein did not decrease until after 2 days of OM treatment (data not shown). The decrease in *p53* mRNA was maximal at 24 h in H3922 cells. These results suggest that either c-Myc is not involved in the regulation of *p53* by OM or it is not the major transcriptional regulator. Other transcription factors could be involved in addition to c-Myc for the OM-induced down-regulation of *p53*.

Two major signaling pathways can be activated by OM and its related cytokines IL-6 and LIF: (a) the Janus family tyrosine kinase/signal transducer and activator of transcription pathway (23); and (b) the Ras-MAP kinase pathway (21, 24–26). In OM-treated breast cancer cells, both

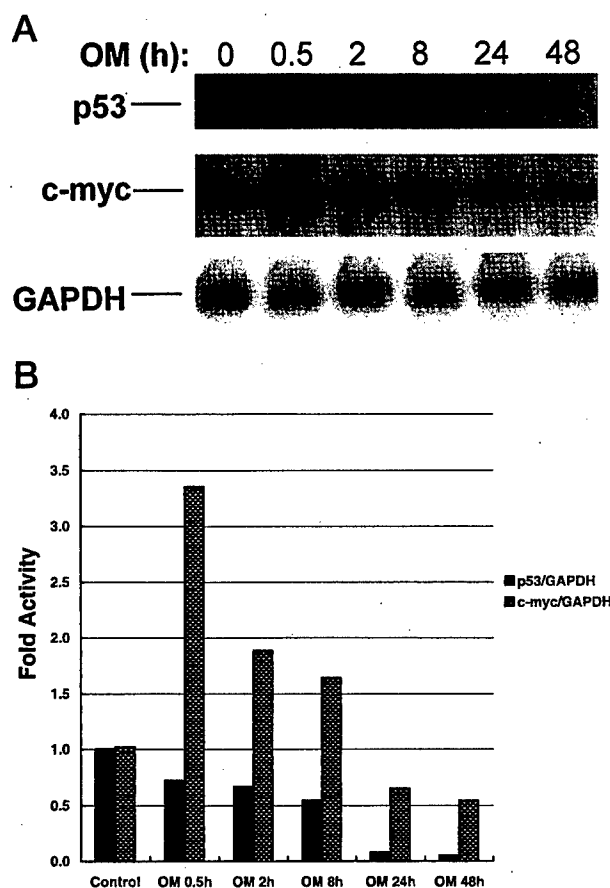


Fig. 7. OM regulates the mRNA expressions of *p53* and *c-myc* with different kinetics. H3922 cells were incubated with 50 ng/ml OM for different times as indicated. Total RNA was isolated and 15 μ g per sample were analyzed for *p53* mRNA, *c-myc* mRNA, and *GAPDH* mRNA by Northern blot. The radioactive signals were detected and quantitated by a Phosphorimager. The figure shown (A) is representative of two different Northern blots. B, the normalized *c-myc* and *p53* mRNA levels (% of control).

STAT3 and MAP kinases ERK1 and ERK2 were activated (27). To investigate whether the MAP kinase pathway is involved in the OM-mediated suppression of p53, H3922 cells were incubated with OM for 2 days in the presence of different concentrations of PD98059, which specifically blocks ERK activation by inhibiting the enzymatic activity of the ERK upstream kinase, MEK (28). Incubation of cells with PD98059 alone, in up to a 30- μ M concentration, did not change the levels of p53 protein (data not shown); however, the OM-mediated suppression of p53 protein expression was partially (maximal 50–60%) inhibited by PD98059 in a dose-dependent manner (Fig. 8). These results suggest that the suppression of p53 expression is a downstream event of the activation of MAP kinase pathway by OM in breast cancer cells. The fact that the OM-inhibitory activity could not be completely reversed by PD98059 at concentrations that effectively inhibited ERK activation (21, 29) suggests that there are other signaling pathways, such as the STAT pathway, that may be involved.

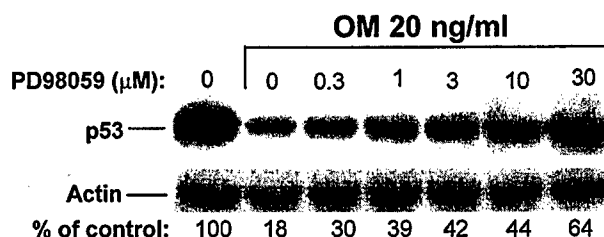


Fig. 8. The dose-dependent effects of MEK inhibitor PD98059 on OM regulation of p53 protein expression. H3922 cells were treated with OM for 2 days in the absence or the presence of the indicated concentrations of PD98059. Total lysate was harvested and analyzed for p53 protein by Western blot. The membrane was stripped and reprobed with anti- β -actin monoclonal antibody. The normalized p53 protein levels expressed as % of control were determined by densitometric analysis of the immunoblot.

Discussion

Previous studies have established a differentiative role of OM in breast cancer cells (13–16). The general effects of OM on breast cancer cells include: (a) inhibition of cellular proliferation in monolayer culture and inhibition of colony formation in soft agar; (b) regulation of the cell cycle by increasing the proportion of cells in G_0 - G_1 phase with a concomitant decrease in the number of cells in S phase; and (c) induction of a variety of morphological changes associated with the differentiated phenotype. These effects are believed to be mediated through the OM-specific receptor that consists of gp130 as a low-affinity ligand-binding subunit and OSMR β as the signal-transducing subunit (13, 19). In this study, we demonstrated that OM also down-regulates p53 expression in breast cancer cells.

The effect of OM on p53 protein expression was initially examined in four breast cancer cell lines, among which 3 cell lines—H3922, MCF-7, and MDA-MB231—were growth-inhibited by OM. The cell line H3477 does not respond to OM treatment because of its lack of expression of the signal-transducing subunit (OSMR β) of the OM-specific receptor. The incubation of cells with OM decreased the level of p53 protein in all three of the OM-responsive cell lines, with the most dramatic effect found in H3922 cells. The expression of p53 protein in H3477 cells was not inhibited at all by OM. The decrease in p53 protein was detected after 9 h of OM treatment and reached the lowest levels (10–20% of control in H3922 and 30–40% of control in MCF-7 and MDA-MB231) after 3–4 days. The extent of the suppression of p53 expression in different cell lines seems to correlate with the expression level of OSMR β , inasmuch as a previous study (19) using quantitative reverse transcription-PCR showed that H3922 cells express the highest mRNA level of OSMR β as compared with that detected in other cell lines.

Northern blot analysis to examine *p53* mRNA expression in control and OM-treated cells demonstrated that OM down-regulated *p53* mRNA expression to a degree similar to that observed in the levels of p53 protein in these cells. The decreased mRNA expression was due to a direct inhibition of transcription of the *p53* gene by OM as demonstrated by nuclear run-on analysis. Collectively, these data establish that OM down-regulates the transcription of the *p53* gene in breast cancer cells, with a resulting decrease in p53 protein

level. The inhibitory effect of OM on *p53* promoter activity suggests that an OM-responsive element(s) is present in the promoter region of the *p53* gene. Interactions of this putative *cis*-acting element with an OM-inducible transcription factor may be responsible for the suppression of *p53* transcription. The nature of this interaction has not been characterized in the present study; however, the fact that the MEK inhibitor PD98059 partially prevented the OM-inhibitory effect on *p53* expression suggests that a substrate of ERK may be directly or indirectly involved in the OM-elicited signaling pathway that mediates this regulation.

It has been proposed that *p53* is a target gene of *c-Myc*. The *c-Myc* protein was reported to transactivate the *p53* promoter through the *c-Myc*-responsive element, E box (CATGTG) (22). Because OM has been shown to regulate *c-myc* gene transcription in breast cancer cells and in M1 leukemia cells, one potential mechanism of OM suppression of *p53* transcription would be suppression of *c-myc* transcription. However, the kinetics of the OM-induced down-regulation of *p53* were different from the kinetics of the OM-induced down-regulation of the *c-myc* gene in these cells. The *c-myc* mRNA was transiently induced by OM within 0.5–8 h and was subsequently suppressed at later time points. The maximal suppression occurred after 2–3 days of OM treatment (Fig. 7; Refs. 13, 14). In contrast, *p53* mRNA was not induced by OM at any time point examined. Instead, it was gradually decreased during the period of OM treatment. A significant decrease in *p53* mRNA level was observed after 8 h of OM treatment. This difference in kinetics between the expression of *p53* mRNA and *c-myc* mRNA suggests that the effect of OM on *p53* transcription is not a direct effect of OM on *c-myc* transcription alone. Additional studies to identify the *cis*-acting element in the *p53* promoter that is responsible for the OM-mediated suppression of *p53* transcription will clarify the relationship between *p53* transcription and *c-myc* transcription in breast cancer cells.

The *p53* tumor suppressor protein is involved in several central cellular processes that are critical for maintaining cellular homeostasis, including gene transcription, DNA repair, cell cycling, senescence, and apoptosis (18, 30). Compared with the vast information and knowledge available regarding the role of *p53* protein in apoptosis, the function of *p53* in cell differentiation is not well understood. This study is the first report to show that *p53* expression is down-regulated in growth-inhibited and -differentiated breast cancer cells through a transcriptional mechanism. Additional studies to examine the functional role of *p53* in the differentiation of breast cancer cells will be needed for a better understanding of the complexity of *p53* functions that are essential for maintaining cell homeostasis.

Materials and Methods

Cells and Reagents. The human breast cancer cell line H3922 was derived from an infiltrating ductal carcinoma, and the human breast cancer cell line H3477 was derived from primary solid tumor (13). MDA-MB231 and MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA). All of the cell lines were cultured in IMDM supplemented with 10% heat-inactivated FBS. Purified human recombinant OM was provided by Bristol-Myers Squibb (Princeton, NJ). PMA and anti- β -actin monoclonal antibody were purchased from Sigma Chemical

Co. (St. Louis, MO). The MEK inhibitor PD98059 was purchased from New England Biolabs (Beverly, MA). The antibodies against *p53* and *c-Myc* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western Blot Analysis of *p53* Protein. Cells were cultured in 60-mm culture plates in 2% FBS IMDM with or without OM. Cells were rinsed with cold PBS and lysed with 0.25 ml of lysis buffer [50 mM Tris (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 5 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1.25 μ g/ml pepstatin, 1 mM Na_3VO_4 , 10 μ M okadaic acid, and 10 μ M cypermethrin]. Concentration of soluble protein from total cell lysate was determined using BCA reagent with BSA as a standard (Pierce). Approximately 10–50 μ g of protein of total cell lysate per sample was separated on 10% SDS PAGE, transferred to PVDF membrane, blotted with anti-*p53* monoclonal antibody (DO-1, Santa Cruz Biotechnology) using an enhanced chemiluminescence (ECL) detection system (Amersham). Membranes were stripped and reblotted with anti- β -actin monoclonal antibody to ensure that an equal amount of protein is loaded on gel. The signals were quantitated with a Bio-Rad Fluoro-S Multimager system. Densitometric analysis of autoradiographs in these studies included various exposure times to ensure linearity of signals.

RNA Isolation and Northern Blot Analysis. Cells were lysed in Ultraspec RNA lysis solution (Biotecx Laboratory, Houston, TX), and total cellular RNA was isolated according to the vendor's protocol. Approximately 15 μ g of each total RNA sample were used in Northern blot analysis as described previously. The *p53* mRNA was detected with a 2-kb ^{32}P -labeled human *p53* cDNA probe, the *c-myc* mRNA was detected with a 1.4-kb probe containing *c-myc* exons 2 and 3, and the *GAPDH* mRNA was detected with a plasmid containing a human *GAPDH* cDNA. Differences in hybridization signals of Northern blots were quantitated by a PhosphorImager.

Nuclear Run-On Analysis. Reaction was performed with the method described previously (14). Briefly, the nuclei were harvested from H3922 cells that were untreated or treated with 50 ng/ml OM for 24 h. Approximately 2×10^7 nuclei in 100 μ l were mixed with 100 μ l of $2\times$ reaction buffer containing 250 μCi [^{32}P]rUTP. Approximately 2.0×10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham) slot blot. Each slot blot contained 5 μ g of *GAPDH* plasmid and 3 μ g of the 2-kb fragment of the *p53* cDNA as described in the Northern blot analysis. Probing the *GAPDH* plasmid allowed normalization of the *p53* signals measured by densitometry.

Transient Transfection Assays. The *p53* promoter luciferase-reporter construct p53Ex1aLUC was generously provided by Dr. Peter Gruss at the Max-Planck-Institute (Göttingen, Germany). For construction of pGL3-p53LUC, the 550-bp insert containing the human *p53* promoter region and Exon 1 (31) was released from p53Ex1aLUC by *KpnI* and *BglII* digestion and subcloned into pGL3-basic digested with *KpnI* and *BglII*. The plasmid vector pDLR234LUC has been described previously (21). H3922 cells, seeded in 12-well tissue culture plates, were transiently transfected with plasmid DNAs pGL3-p53LUC and pDLR234LUC by the method of calcium phosphate coprecipitation. Fifteen h after transfection, OM (50 ng/ml) was added. After a 48-h treatment, cells were lysed. Equal amounts of cell lysate from each well were used for measuring luciferase activity. The data (mean \pm SE) shown are representative of three separate experiments in which triplicate wells were assayed (Fig. 6).

Acknowledgments

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References

- Zarling, J. M., Shoyab, M., Marquardt, H., Hanson, M. B., Lionbin, M. N., and Todaro, G. J. Oncostatin M: a growth regulator produced by differentiated lymphoma cells. *Proc. Natl. Acad. Sci. USA*, 83: 9739–9743, 1986.
- Brown, T. J., Lionbin, M. N., and Marquardt, H. Purification and characterization of cytostatic lymphokines produced by activated human T-lymphocytes: synergistic antiproliferative activity of transforming growth factor β 1, interferon γ , and oncostatin M for human melanoma cells. *J. Immunol.*, 139: 2977–2983, 1987.

3. Grove, R. I., Mazzucco, C. E., Allegretto, N., Kiener, P. A., Spitalny, G., Radka, S. F., Shoyab, M., Antonaccio, M., and Warr, G. A. Macrophage-derived factors increase low-density lipoprotein uptake and receptor number in cultured human liver cells. *J. Lipid Res.*, 32: 1889–1897, 1991.
4. Rose, T. M., and Bruce, A. G. Oncostatin M is a member of a cytokine family that includes leukemia inhibitory factor, granulocyte colony stimulating factor, and interleukin 6. *Proc. Natl. Acad. Sci. USA*, 88: 8641–8645, 1991.
5. Bazan, F. Neurotrophic cytokines in the hematopoietic fold. *Neuron*, 7: 197–208, 1991.
6. Pennica, D. Cardiotrophin-1: biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J. Biol. Chem.*, 270: 10915–10922, 1998.
7. Ip, N. Y., Nye, S., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stäh, N., and Yancopoulos, G. CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducer receptor component gp130. *Cell*, 69: 1121–1132, 1992.
8. Horn, D., Fitzpatrick, W. C., Gompfer, P. T., Ochs, V., Bolton-Hanson, M., Zarling, J. M., Malik, N., Todaro, G. J., and Linsley, P. S. Regulation of cell growth by recombinant oncostatin M. *Growth Factors*, 2: 157–165, 1990.
9. Liu, J., Clegg, J. C., and Shoyab, M. Regulation of *EGR-1*, *c-jun*, and *c-myc* gene expression by oncostatin M. *Cell Growth Differ.*, 3: 307–313, 1992.
10. Grove, R. I., Eberhardt, C., Abid, S., Mazzucco, C. E., Liu, J., Todaro, G. J., Kiener, P. A., and Shoyab, M. Oncostatin M is a mitogen for rabbit vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA*, 90: 823–827, 1993.
11. Zhang, X. G., Gu, J. J., Lu, Z. Y., Yasukawa, K., Yancopoulos, G. D., Turner, K., Shoyab, M., Taga, T., Kishimoto, T., Bataille, R., and Klein, B. Ciliary neurotrophic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. *J. Exp. Med.*, 179: 1343–1347, 1994.
12. Nair, B. C., DeVico, A. L., Nakamura, S., Copeland, T. D., Chen, Y., Patel, A., O'Neil, T., Oroszian, S., and Gallo, R. C. S. M. G. Identification of a major growth factor for AIDS-Kaposi's sarcoma cell as oncostatin M. *Science (Washington DC)*, 255: 1430–1432, 1992.
13. Liu, J., Spence, M. J., Wallace, P. M., Forcier, K., Hellstrom, I., and Vestal, R. E. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth and down-regulation of the *c-myc* proto-oncogene. *Cell Growth Differ.*, 8: 667–676, 1997.
14. Spence, M. J., Vestal, R. E., and Liu, J. Oncostatin M-mediated transcriptional suppression of the *c-myc* gene in breast cancer cells. *Cancer Res.*, 57: 2223–2228, 1997.
15. Douglas, A. M., Grant, S. L., Goss, G. A., Clouston, D. R., Sutherland, R. L., and Begley, C. G. Oncostatin M induces the differentiation of breast cancer cells. *Int. J. Cancer*, 75: 64–73, 1998.
16. Douglas, A. M., Goss, G. A., Sutherland, R. L., Hilton, D. J., Berndt, M. C., Nicola, N. A., and Begley, C. G. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. *Oncogene*, 14: 661–669, 1997.
17. Guilbaud, N. F., Gas, N., DuPont, M. A., and Valette, A. Effects of differentiation-inducing agents on maturation of human MCF-7 breast cancer cells. *J. Cell. Physiol.*, 145: 162–172, 1990.
18. Vogelstein, B., and Kinzler, K. W. p53 function and dysfunction. *Cell*, 70: 523–526, 1992.
19. Liu, J., Hadjokas, N., Mosley, B., Estrov, Z., Spence, M. J., and Vestal, R. E. Oncostatin M-specific receptor expressions and function in regulating cell proliferation of normal and malignant mammary epithelial cells. *Cytokine*, 10: 295–302, 1998.
20. Bacus, S. S., Kiguchi, K., Chin, D., King, C. R., and Huberman, E. Differentiation of cultured human breast cancer cells (AU-565 and MCF-7) associated with loss of cell surface HER-2/neu antigen. *Mol. Carcinog.*, 3: 350–352, 1990.
21. Li, C., Kraemer, F. B., Ahlborn, T. E., and Liu, J. Induction of low density lipoprotein receptor (LDLR) transcription by oncostatin M is mediated by the extracellular signal-regulated kinase signaling pathway and the repeat 3 element of the LDLR promoter. *J. Biol. Chem.*, 274: 6747–6753, 1999.
22. Roy, B. B. J., Balint, E., and Reisman, D. Transactivation of the human p53 tumor suppressor gene by c-myc/max contributes to elevated mutant p53 expression in some tumors. *Mol. Cell. Biol.*, 14: 7805–7815, 1994.
23. Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N., and Yancopoulos, G. D. Association and activation of Jak-Tyk kinase by CNTF-LIF-OSM-IL-6 receptor components. *Science (Washington DC)*, 263: 92–95, 1994.
24. Thoma, B., Bird, T. A., Friend, D. J., Gearing, D. P., and Dower, S. K. Oncostatin M and leukemia inhibitory factor trigger overlapping and different signals through partially shared receptor complexes. *J. Biol. Chem.*, 269: 6215–6222, 1994.
25. Yin, T., and Yang, Y. C. Mitogen-activated protein kinases and ribosomal S6 protein kinases are involved in signaling pathways shared by interleukin-11, interleukin-6, leukemia inhibitory factor, and oncostatin M in mouse 3T3-L1 cells. *J. Biol. Chem.*, 269: 3731–3738, 1994.
26. Stancato, L. F., Yu, C. R., Petricoin, E. F., III, and Lerner, A. C. Activation of Raf-1 by interferon γ and oncostatin M requires expression of the Stat1 transcription factor. *J. Biol. Chem.*, 273: 18701–18704, 1998.
27. Liu, J., Li, C., Ahlborn, T. E., and Kraemer, F. B. Oncostatin M-induced growth inhibition and differentiation of breast cancer cells is mediated by the MAP kinase ERK signaling pathway. *Proc. Am. Assoc. Cancer Res.*, 90: 334, 1999.
28. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.*, 270: 27489–27494, 1995.
29. Kumar, A., Chambers, T. C., Cloud-Heflin, B. A., and Mehta, K. D. Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44 MAP kinase activation. *J. Lipid Res.*, 38: 2240–2248, 1997.
30. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell*, 88: 323–331, 1997.
31. Stuart, E. T., Haffner, R., Oren, M., and Gruss, P. Loss of p53 function through PAX-mediated transcriptional repression. *EMBO J.*, 14: 5638–5645, 1995.



The critical role of the PE21 element in oncostatin M-mediated transcriptional repression of the p53 tumor suppressor gene in breast cancer cells

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Cytokine oncostatin M (OM) exerts growth-inhibitory and differentiative effects on breast cancer cells. Previously we showed that the transcription from the p53 gene in breast cancer cells was down regulated by OM. To elucidate the molecular mechanisms underlying the OM effect on p53 transcription, in this study, we dissected the p53 promoter region and analysed the p53 promoter activity in breast tumor cells. We showed that treatment of MCF-7 cells with OM induced a dose- and time-dependent suppression of p53 promoter activity. The p53 promoter activity was decreased to 35% of control at 24 h and further decreased to 20% at 48 h by OM at concentrations of 5 ng/ml and higher. Deletion of the 5'-flanking region of the p53 promoter from –426 to –97 did not affect the OM effect. However, further deletion to –40 completely abolished the repressive effect of OM. The p53 promoter region –96 to –41 contains NF- κ B and c-myc binding sites, and a newly identified UV-inducible element PE21. Mutations to disrupt NF- κ B binding or c-myc binding to the p53 promoter decreased the basal promoter activity without affecting the OM-mediated suppression, whereas mutation at the PE21 motif totally abolished the OM effect. We further demonstrated that insertion of PE21 element upstream of the thymidine kinase minimal promoter generated an OM response analogous to that of the p53 promoter. Finally, we detected the specific binding of a nuclear protein with a molecular mass of 87 kDa to the PE21 motif. Taken together, we demonstrate that OM inhibits transcription of the p53 gene through the PE21 element. Thus, the PE21 element is functionally involved in p53 transcription regulated by UV-induction and OM suppression. *Oncogene* (2001) 20, 8193–8202.

Keywords: p53 tumor suppressor gene; transcriptional regulation; oncostatin M

Introduction

The p53 tumor suppressor protein is involved in several central cellular processes that are critical for maintaining cellular homeostasis, including gene transcription (Nakano *et al.*, 2000; Xu *et al.*, 2000), DNA repair (Kao *et al.*, 2000; Zhu *et al.*, 2000), cell cycling (Jeffy *et al.*, 2000; Hirose *et al.*, 2001), senescence (Peeper *et al.*, 2001; Seluanov *et al.*, 2001), and apoptosis (Uberti and Grilli, 2000; Zeng *et al.*, 2000). Compared to the vast information and knowledge available regarding the regulation of p53 protein expression and function (Wang and Friedman, 2000; Ito *et al.*, 2001; Wang *et al.*, 2001), there is only a small amount of literature on transcriptional regulation of the p53 gene (Balint and Reisman, 1996; Benoit *et al.*, 2000; Mokdad-Gargouri *et al.*, 2001). However, control of p53 gene expression at the transcriptional level has been shown to play important roles in mitogenic stimulation or factor induced differentiation (Reich and Levine, 1984; Soini *et al.*, 1992). Moreover, the deregulated transcription of p53 accounts for at least in part, the elevated expression of mutant p53 in tumor cells (Balint and Reisman, 1996).

Since cloning of the human p53 promoter in 1989 (Krawford and Crawford, 1989), several transcription factors have been identified that interact with specific regions of the p53 promoter to positively or negatively regulate transcription. The transcription factors shown to positively regulate p53 transcription include c-myc (Kirch *et al.*, 1999; Lee and Rho, 2000), NF- κ B (Pei *et al.*, 1999; Benoit *et al.*, 2000), YY1/NF1 (Lee *et al.*, 1998, 1999; Nayak and Das, 1999), Ap1 (Kirch *et al.*, 1999), and the HoxA5 homeobox containing gene product (Raman *et al.*, 2000). Members of the PAX family are the only mammalian nuclear proteins shown to repress p53 transcription through a binding site present in the first non-coding exon (Stuart *et al.*, 1995).

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Recently, a novel 21 bp motif, named the PE21 element, was identified in the human p53 promoter that is located immediately upstream of the NF- κ B binding site (Noda *et al.*, 2000). It was shown that the PE21 element covering the region of -79 to -59 is a primary determinant for the basal transcription of the p53 gene and the sequence required for UV-induced transcription in human fibroblasts. Mutations within this region drastically reduced the basal promoter activity and abolished the UV-induction. Interestingly, this 21 bp motif appears to have a function in initiation of transcription in a bi-directional manner. Insertion of multiple copies of PE21 in the sense or antisense orientation into a promoterless luciferase reporter pGL2-basic initiated the transcription of the luciferase gene and generated an UV-inducible response as well. It remains to be elucidated whether the PE21 element has a functional role in p53 transcription regulated by cellular factors or other extracellular stimuli. Furthermore, the PE21 binding proteins need to be identified and characterized.

Oncostatin M (OM), a 28 kDa glycoprotein, is a cytokine produced by activated T lymphocytes and macrophages (Zarling *et al.*, 1986). Previous studies showed that OM inhibits the growth of several breast cancer cell lines, including MCF-7, MDA-MB231, and H3922, which is a cell line derived from an infiltrating ductal carcinoma (Horn *et al.*, 1990; Douglas *et al.*, 1997, 1998; Liu *et al.*, 1997; Spence *et al.*, 1997). Breast cancer cells respond to OM treatment with reduced growth rates and the appearance of differentiated phenotypes. However, OM treatment does not appear to lead to apoptosis. Since the p53 tumor suppressor protein plays important roles in cellular proliferation and differentiation, we examined the effects of OM on p53 expression in breast cancer cells. Surprisingly, we found that p53 expression was down regulated by OM in MCF-7, MDA-MB231, and H3922 cells (Liu *et al.*, 1999). Decreased levels of p53 protein and mRNA were detected after 1 day of OM treatment and reached maximal suppression of 10–20% of control after 3 days in H3922 cells. Nuclear run-on assays further demonstrated that OM decreased the number of actively transcribed p53 mRNA. These studies suggest that OM may repress p53 gene transcription. The effect of OM on p53 transcription appears to precede its effects on cell growth inhibition and induction of morphological changes, as the retardation of cell growth by OM as measured by [3 H]-thymidine incorporation could be detected after 2 days but a decrease in the level of p53 mRNA could be detected as early as 6–8 h.

In order to delineate the molecular mechanisms by which OM regulates p53 transcription and to understand the relationship between p53 expression and proliferation and differentiation of breast cancer cells, in this study we dissected the p53 promoter region to identify the *cis*-acting element that mediates the OM effect in MCF-7 cells. Our results demonstrate that the effect of OM is not mediated through the known repressor PAX binding site. Instead the PE21 element

is responsible for OM-induced suppression of p53 transcription. Mutation of PE21 in the context of the p53 promoter completely abolished the inhibitory activity of OM on p53 transcription. By contrast, insertion of the PE21 motif into an OM-unresponsive TK promoter created a phenomenon of transcriptional suppression, resembling the p53 promoter.

Results

Deletion analysis to define the regulatory sequences involved in the basal transcriptional activity of the p53 promoter in breast tumor cells

The regulatory sequences that control p53 transcription in breast tumor cells have not been clearly defined, although a number of studies had examined p53 promoter activity in other cell types. Thus, initially in the present study, we generated a series of reporter constructs in which luciferase gene is driven by varying lengths of the 5'-flanking region of the p53 gene. These constructs were tested for activity in MCF-7 cells. A diagram of the deletion constructs is shown in Figure 1. Figure 2a compared the basal promoter activity of the deletion constructs with the activity of the full promoter construct pGL3-p53Luc that contains a 599 bp fragment of the p53 promoter (-426 to +172) (Liu *et al.*, 1999). These results, representing 6–8 separate transfections, showed that deletion of the 5'-flanking region from -426 to -177 did not affect the p53 promoter activity, whereas deletion down to -97 (5' Del-4) significantly lowered the basal activity to approximately 40% of the full promoter. Further deletion to -41 (5' Del-5) to eliminate the binding sites for NF- κ B and *c-myc* drastically reduced the basal promoter activity to a level below 5% of the full promoter. These data suggest that the transcription factors NF- κ B and *c-myc* play critical roles in the basal transcriptional activity of the p53 gene in breast tumor cells, however, the promoter region covering -176 to -97 may contain a regulatory sequence that is responsible for the maximal basal transcriptional activity of the p53 gene in MCF-7 cells.

The p53 promoter region from -176 to -97 contains a stretch of CT rich sequence (CCCTCCTCCCC -174 to -164), a potential binding site for the transcription factor Sp1. To determine whether Sp1 interacts with this sequence, electrophoretic mobility shift assay (EMSA) was conducted with the nuclear extract isolated from MCF-7 cells and a 32 P-labeled double-stranded oligonucleotide, p53-Sp1, corresponding to the promoter region -183 to -154. Upon incubation of p53-Sp1 with nuclear extract, two DNA-protein complexes were detected (Figure 2b, lane 1). Formation of these complexes was inhibited by competition with a 100-fold molar excess of the unlabeled probe p53-Sp1 (lane 2), but was not inhibited by the oligonucleotide p53-mSp1 that contains mutations within the CT-stretch (lane 3).

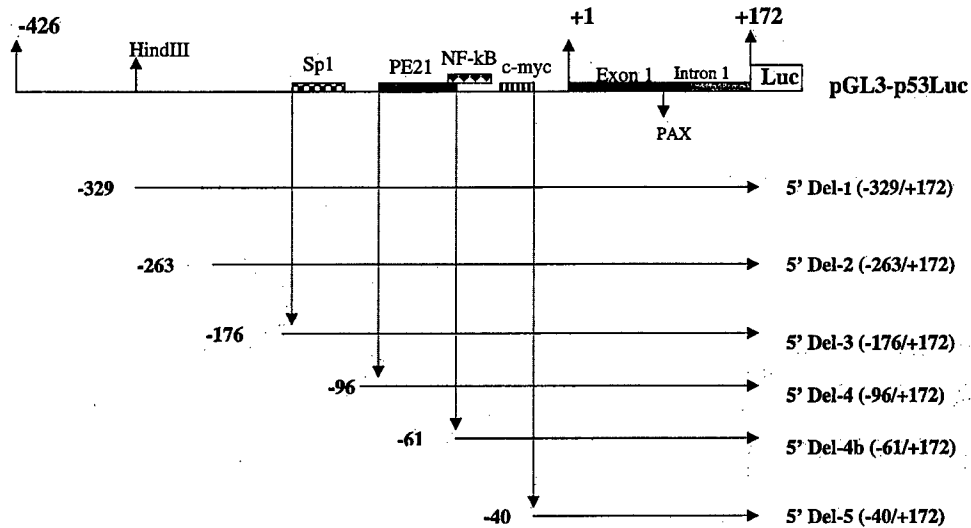


Figure 1 Schematic representation of p53 promoter luciferase reporter plasmid. A 599 bp fragment of the p53 gene covering -426 to $+172$ was inserted into $5'$ *KpnI* and $3'$ *BglII* sites of the promoter-less luciferase reporter pGL3-basic. The $5'$ deletion fragments of the p53 promoter were synthesized by PCR using pGL3-p53Luc as the template. The p53 promoter fragments were inserted into $5'$ *SacI* and $3'$ *XhoI* sites of pGL3-basic. The most $3'$ end of the major transcription initiation site for the human p53 gene is defined as $+1$ and the locations of the $5'$ ends of the promoters are indicated by the negative numbers of nucleotides relative to the transcription start site

The faster moving complex was supershifted by the anti-Sp3 antibody (lane 5), whereas the slower moving complex was partially supershifted by the anti-Sp1 antibody (lane 4). Inclusion of anti-Sp1 and anti-Sp3 antibodies together in the reaction mixture completely supershifted both complexes (lane 6). These data demonstrate that transcription factors Sp1 and Sp3 bind to this CT-rich region of the p53 promoter.

To determine the function of Sp1/Sp3 in mediating the p53 basal promoter activity, the Sp1 site in p53Luc was mutated (CCCTCCTCCCC to CGCTCGTCGCC) and the mutated reporter p53Luc-mSp1 along with the wild type vector p53Luc were transfected into MCF-7 cells. Figure 2c shows that mutation of this Sp1 site lowered the p53 promoter activity by approximately 50%, thereby suggesting that loss of the Sp1/Sp3 binding to the CT-rich region is primarily responsible for the diminished basal promoter activity of the deletion mutant 5' Del-4. These results together demonstrate that Sp1 and Sp3 are positive trans-activators of p53 transcription and that their binding to the CT rich sequence contributes to the basal transcriptional activity of the p53 gene.

Dose-dependent and time-dependent responses of p53 transcription to OM

Next, the effect of OM on p53 promoter activity in MCF-7 cells was examined. The full promoter construct p53Luc was transiently transfected into MCF-7 cells along with the renilla luciferase expression vector pRL-TK. After transfection, cells were untreated or treated with OM at different concentrations for 48 h. Figure 3a shows that the

suppressive effect of OM on p53 promoter activity was detected at 0.1 ng/ml, and a maximal suppression of 75–80% of p53 promoter activity was observed at 5 ng/ml. The inhibitory effect of OM on p53 transcription was also time-dependent. The p53 promoter activity was decreased to 67% of control by 8 h, lowered to 35% by 24 h, and further declined to 20% of control by 48 h after treating cells with a saturable concentration of OM. These results clearly demonstrate that OM represses p53 promoter activity in a dose-dependent and a time-dependent manner that is directly correlated with the effects of OM on p53 mRNA expression, as we previously reported (Liu et al., 1999).

The biological functions of OM can be mediated through two types of receptor complexes, the leukemia inhibitory factor (LIF)/OM shared receptor (type I) and the OM-specific receptor (type II). Previous studies have shown that MCF-7 cells express both the type I and the type II receptors of OM (Estrov et al., 1995). To determine which receptor complex mediates the effect of OM on p53 transcription, we compared the effect of OM with that of LIF on p53 promoter activity. As shown in Figure 4, in contrast to the strong inhibitory effect of OM, LIF at saturable concentrations (50 and 100 ng/ml) had no effect at all on the activity of p53 promoter p53Luc, thereby implying that OM regulates the transcription of the p53 gene mainly through the type II OM-specific receptor not the OM/LIF shared receptor type I complex.

Dissection of the p53 promoter to define the OM-responsive region

To define the OM-responsive region in the p53 promoter, the $5'$ and $3'$ deletion constructs of the p53

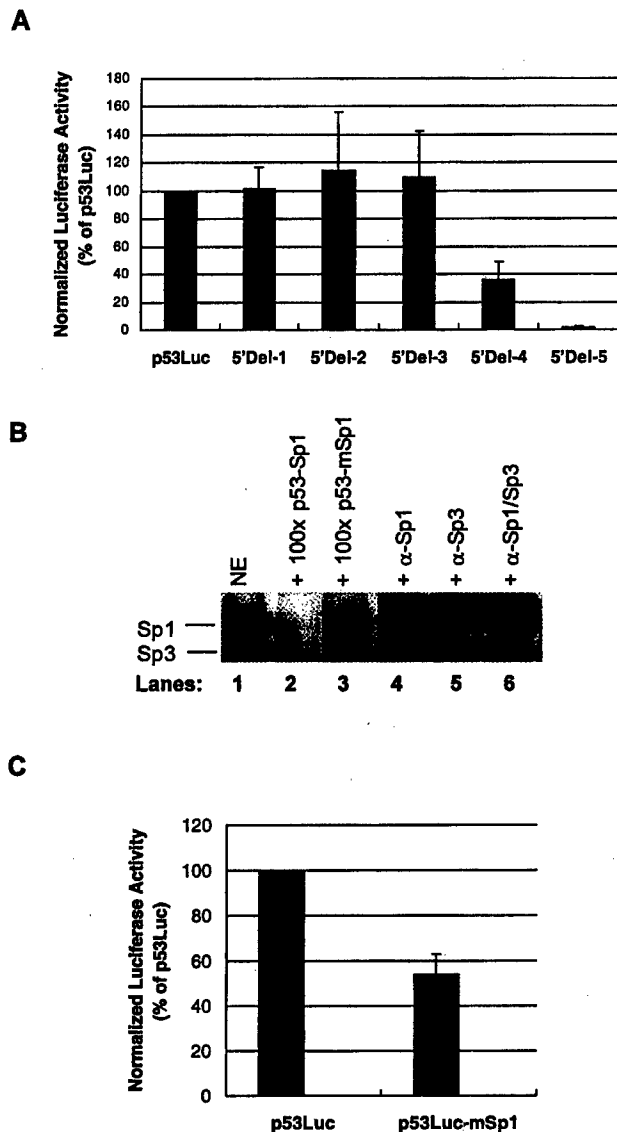


Figure 2 P53 promoter deletion and mutation analysis to define functional regulatory sequences that control the basal promoter activity of the p53 gene. (a) Deletion analysis: p53Luc and 5' deletion constructs containing various lengths of the promoter were transfected into MCF-7 cells along with the vector pRL-TK. Cell lysate was harvested 40 h after transfection. The normalized luciferase activity of p53Luc is expressed as 100%. The data (mean \pm s.d.) shown are derived from 6–8 separate transfection experiments in which triplicate wells were assayed. (b) EMSA: A double stranded oligonucleotide corresponding to the p53 promoter region –183 to –154, designated as p53-Sp1, was radiolabeled and incubated with 10 μ g of nuclear extract prepared from MCF-7 cell in the absence (lane 1) or in the presence of 100-fold molar unlabeled competitors (lanes 2, 3), or in the presence of antibodies (lanes 4–6). The reaction mixture was loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 2.5 h at 4°C. (c) Mutation analysis: p53Luc-wt and p53Luc-mSp1 were transiently transfected into MCF-7 along with the vector pRL-TK. The normalized luciferase activity of p53Luc-wt is expressed as 100%. The p53Luc-mSp1 exhibited 54.1% activity compared with p53Luc-wt.

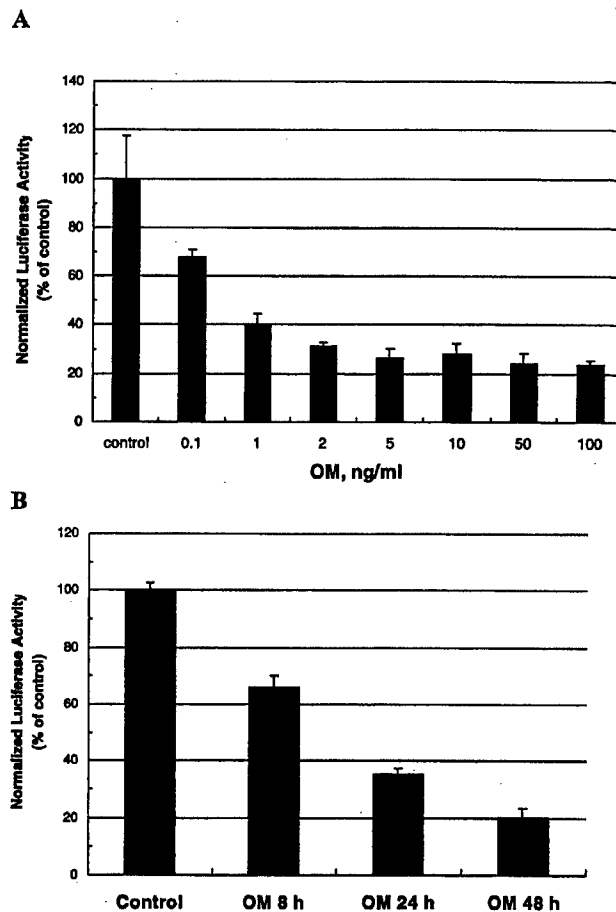


Figure 3 OM down-regulates p53 promoter activity in a dose-dependent and a time-dependent manner. MCF-7 cells were transfected with p53Luc along with the vector pRL-TK. (a) After addition of DNA into the medium, OM dilution buffer or OM at different concentrations were added to the cells and cells were harvested 40 h later. (b) After addition of DNA into the medium, OM at a saturable concentration (50 ng/ml) was added to the cells at different times and cells were harvested together after 48 h of transfection. The normalized luciferase activity of transfected cells that were untreated is expressed as 100%. The data (mean \pm s.d.) shown are representative of three independent transfection experiments in which triplicate wells were transfected for each condition.

promoter were transfected into MCF-7 cells. Then the transfected cells were untreated or treated with OM for 40 h prior to cell lysis. The results of 6–8 transfection assays using 5' deletion constructs are summarized in Figure 5. These results showed that deletion of the 5'-flanking region from –426 to –97 did not affect the OM response. In contrast, further deletion to –41 (5' Del-5) eliminated the OM effect. These data suggest that the promoter region covering –96 to –40 is not only important for the basal transcriptional activity as shown in Figure 2a but it may also contain the critical OM-responsive element. Furthermore, shortening of the 3' region from +172 to +14 to delete the PAX binding site had no effect on OM-mediated suppression or the basal promoter activity, thereby excluding the involvement of the repressor PAX in OM-mediated down regulation of p53 transcription (data not shown).

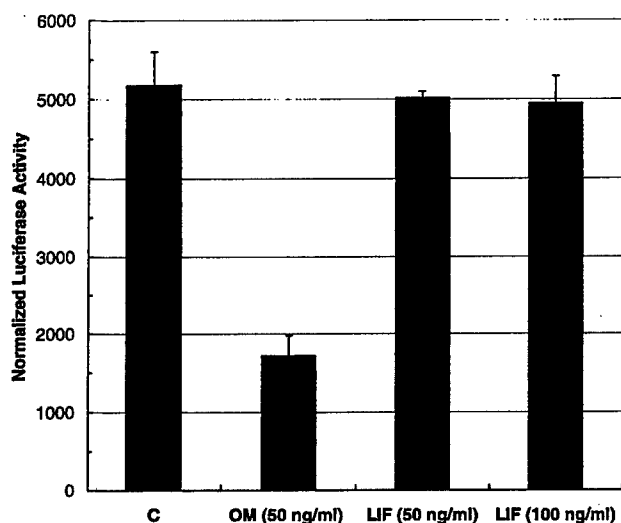


Figure 4 Comparison of inhibitory effect of OM and LIF on p53 promoter activity. MCF-7 cells were transfected with p53Luc along with the vector pRL-TK. After addition of DNA into the medium, OM or LIF at indicated concentrations were added to the cells and cells were harvested 40 h later

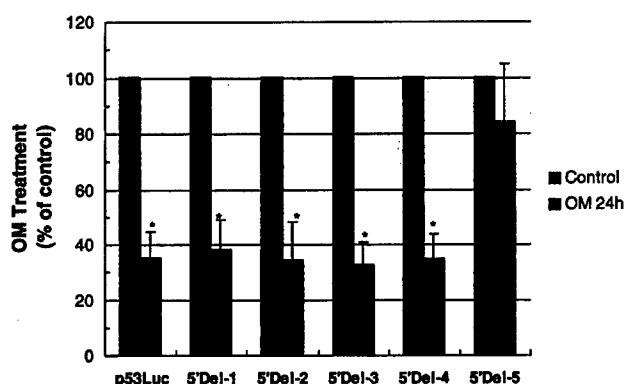


Figure 5 The proximal region of the p53 promoter contains an OM-responsive element. P53Luc and 5' deletion constructs containing various lengths of the promoter were transfected into MCF-7 cells along with the vector pRL-TK. After transfection, cells were incubated in the presence or absence of OM 50 ng/ml for 24 h. The normalized luciferase activity of transfected cells that were untreated is expressed as 100%. The data (mean \pm s.e.) shown were derived from 6–8 independent transfection assays. Differences in normalized luciferase activities between untreated and OM treated samples were evaluated using two tailed Student's *t*-test. A statistically significant difference ($P < 0.05$) is indicated by an asterisk

Localization of the OM-responsive sequence to the PE21 element

The 5' deletion analysis localized the OM-responsive sequence to the proximal region of the p53 promoter from -96 to -41. This region contains three important regulatory motifs including NF- κ B, the E-box (*c-myc*), and the newly identified UV-inducible PE21 element. To investigate the role of these regulatory sequences in OM-mediated suppression, site-directed mutagenesis was conducted on the full promoter p53Luc to mutate each binding site indi-

dually. Figure 6 shows that mutation of the *c-myc* site lowered the basal promoter activity 75% without affecting the OM effect. Likewise, mutation of the NF- κ B binding site decreased the basal promoter activity 85% with little effect on OM. By contrast, mutation at the PE21 element drastically reduced the basal promoter activity and rendered the p53 promoter unresponsive to OM. To confirm this finding, the PE21 element was mutated in the vector 5' Del-4 that contains the minimal sequence for the basal transcription and the OM-mediated suppression. Again, the OM inhibitory effect was not observed in the PE21 mutant in the context of this short promoter fragment. Similarly, the suppressive effect of OM was not seen on the plasmid 5' Del-4b in which the PE21 element was deleted.

Next, we were interested in determining whether OM could exert its effect on PE21 in the context of a heterologous promoter that contains the PE21 element without auxiliary sequences of p53 promoter. To test this, luciferase reporters containing different copies of PE21 in tandem inserted 5' upstream of a minimal HSV tk promoter (pTKLuc) in either sense, pTKLuc-PE21 (S), or antisense, pTKLuc-PE21 (As) orientations were transfected into MCF-7 cells. The plasmid pTKLuc produced low but measurable luciferase activity in MCF-7 cells and OM treatment did not lower the activity. Inclusion of the PE21 sequence in either direction greatly increased luciferase activities from 20–400-fold of the pTKLuc. The fold increase of luciferase activity was correlated with the increase in PE21 copy number in most cases and showed a preference with the antisense orientation. Importantly, in contrast to the vector pTKLuc, the reporters containing the PE21 element clearly displayed responses to OM with luciferase activities reduced to 39 to 59% of control in the OM treated cells (Figure 7), comparable to the OM effect observed in the native p53 promoter. Together, our results presented in Figures 6 and 7 provide strong evidence that the PE21 element plays an important role in the basal transcription of the p53 gene and is also critically involved in the OM-induced transcriptional suppression of the p53 gene in breast cancer cells.

Characterization of nuclear proteins that interact with the PE21 element

To detect nuclear proteins in MCF-7 cells that specifically interact with the PE21 sequence, EMSA was conducted with 32 P-labeled oligonucleotide p53-PE21 containing the PE21 and flanking sequence, and nuclear extracts prepared from untreated or OM 40 h-treated cells. Figure 8 shows that three specific complexes were detected in both control and OM-treated nuclear extracts. The formation of these complexes was inhibited with 100-fold molar excess of unlabeled probe (lanes 2, 7) but was not inhibited by a 100-fold molar excess of an unrelated DNA containing an estrogen response element (lanes 5, 10). An oligonucleotide containing the NF- κ B site of the p53

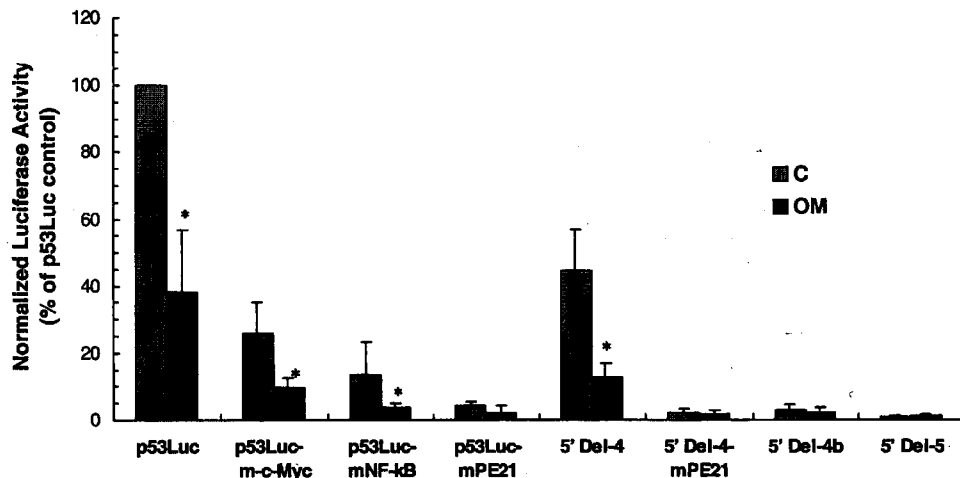


Figure 6 Localization of the OM response to the PE21 element in the human p53 promoter. P53 promoter reporter wild type and mutants were transfected into MCF-7 cells individually. After transfection, cells were incubated in the presence or absence of OM (50 ng/ml) for 40 h. The data represent the results of 6–8 independent transfections. The normalized luciferase activity of each vector was expressed as the per cent of luciferase activity of p53Luc wild type vector in untreated control cells. An asterisk sign indicates that there is a statistically significant difference between the untreated control and the OM treated sample

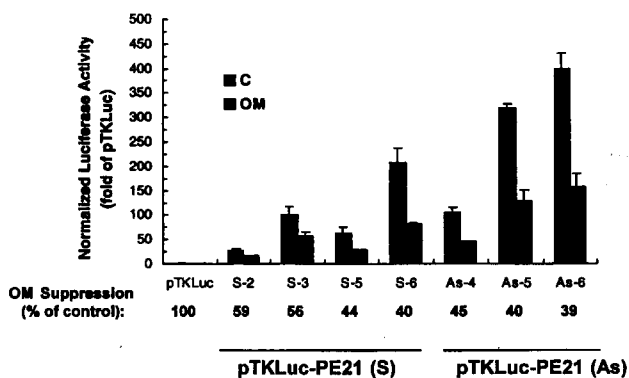


Figure 7 Effects of OM on heterologous promoter constructs containing the PE21 element. The pTKLuc-PE21 vectors were constructed by insertion of sequences of the PE21 element in tandem in sense (S) or antisense (As) orientation adjacent and upstream of the TATA box from the HSV tk promoter. The number indicates the number of repeat of the PE21 element in each construct. These vectors were transiently transfected into MCF-7 cells and examined for responses to OM treatment as described in Figure 5. The data shown are representative of 3–4 separate transfections

promoter competed for the binding of complex A but not complexes B and C (lanes 4, 9). The binding of complex A was also competed by oligonucleotide p53-mPE21 that contains a 3 bp mutation within the PE21 sequence (lanes 3, 8). These data suggest that the complex A was formed at sequences that flank the PE21 core element. Thus, the identity of complex A was not further investigated in this study since the emphasis of this study is on the PE21 core element.

Complexes B and C are PE21 specific as the oligonucleotide p53-mPE21 lost the ability to compete with the binding of these two complexes to the labeled PE21 probe. Apparently OM treatment of 40 h did not altered the pattern of the complexes or the intensity of

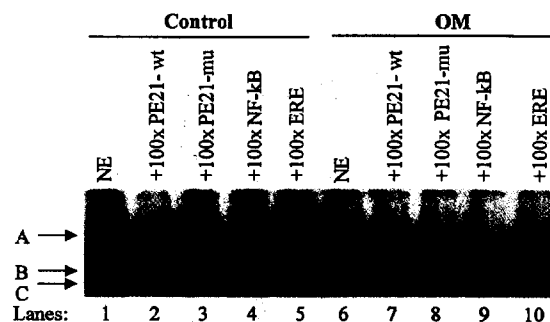


Figure 8 EMSA analysis of nuclear proteins interacting with the PE21 motif. A double stranded oligonucleotide, designated as p53-PE21, was radiolabeled and incubated with 10 μ g of nuclear extracts prepared from untreated (lanes 1–5) or OM 40 h treated MCF-7 cell (lanes 6–10) in the absence (lanes 1, 6) or in the presence of 100-fold molar unlabeled competitors. The reaction mixture was loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 2.5 h at 4°C

the binding signals. Similar results were obtained with the nuclear extracts treated with OM for different length of times including 6 and 24 h. These observations were consistent with previous results of UV-induction. It was shown that the binding of nuclear proteins of fibroblasts without or with UV-irradiation to the PE21 probe was not different (Noda *et al.*, 2000).

Previous studies conducted in human fibroblasts did not characterize the protein/DNA complex of the PE21 sequence. It is unknown whether a single DNA binding protein or multiple proteins interact with the PE21 motif. To characterize the MCF-7 nuclear proteins present in the PE21 DNA complexes, EMSA with MCF-7 nuclear extract and the labeled PE21 probe was followed by UV cross-linking. Complex B was excised from the gel and the protein components were analysed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Analysis of the SDS-PAGE revealed that one protein was crosslinked to the labeled PE21 probe (Figure 9). After correction for the bound oligonucleotide, the molecular mass of the protein appeared to be 87 kDa. A similar procedure was used to characterize complex C, but the UV-crosslinking experiments failed to detect any proteins present in the complex C, probably due to the low abundance of the complex and low efficiency of the UV-crosslinking.

Discussion

The PE21 element was originally discovered by searching for the sequence that was responsible for the UV-induced transcription of the p53 gene in human fibroblasts (Noda *et al.*, 2000). Intriguingly, in this study, we found that the repressive effect of OM on p53 transcription was also mediated through this regulatory element. Our studies further highlight the importance of the PE21 element in the control of p53 gene transcription.

Our studies clearly demonstrate that the PE21 element is a critical sequence that controls p53 transcription in breast cancer cells, as mutation of this sequence produced the severest impact on p53 promoter activity in MCF-7 cells as compared to mutations on other functional sites such as NF- κ B or the bHLH *c-myc* binding site. Mutations to disrupt the binding of NF- κ B to its recognition sequence adjacent to PE21 lowered the basal promoter activity to 15% of the wild type promoter; mutation of the basic HLH site to interfere the binding of *c-myc* reduced the basal transcription by 75%. In contrast, alteration of 3 bp within the PE21 motif nearly eliminated the transcrip-

tional activity of the p53 promoter. The promoter activity with the PE21 mutant was less than 5% of the wild type promoter. The ability of PE21 in activation of gene transcription was further demonstrated by its strong inducing effect on pTKLuc that contains a weak promoter composed a TATA box and one non-functional Sp1 site.

In this study we showed that mutations of the PE21 element in the context of the full p53 promoter (p53Luc) or in the context of the short promoter fragment (5' Del-4) that retains 40% of the p53 promoter activity completely eliminated the inhibitory effect of OM on p53 transcription. In contrast, mutations at other functional sites including NF- κ B or *c-myc* did not abolish the OM inhibitory activity. The critical role of the PE21 site in OM-mediated repression of p53 transcription is further supported by the results of transfection with the pTKLuc-PE21 reporters. OM had no effect on the promoter activity of pTKLuc. However, the promoter activities of pTKLuc-PE21 in either the sense or antisense orientation were clearly suppressed by OM to levels comparable to that observed in the p53 promoter. These results suggest that the PE21 element is the primary *cis*-acting sequence that mediates the OM-induced transcriptional repression of the p53 gene.

We were able to detect two DNA-protein complexes formed with the PE21 sequence from MCF-7 cells. Complex B was relatively more abundant than complex C. EMSA experiments followed by UV-cross linking and SDS-PAGE revealed that a nuclear protein with a molecular mass around 87 kDa was present in complex B. Interestingly, in an effort to purify the PE21 binding proteins, two proteins with molecular weights of 80 and 85 kDa were isolated from Molt 4 cells through PE21-sepharose affinity column. These two proteins were shown to specifically bind to the PE21 probe (Noda *et al.*, unpublished data). It is possible that the 87 kDa protein present in the complex B is identical or related to one of these two PE21 binding proteins. The relationship between complexes B and C is presently unknown. It is highly likely that more than one nuclear protein binds to the PE21 sequence. Alternatively, the faster moving complex C may represent a degradation product of the protein in complex B.

OM treatment did not alter the binding of nuclear proteins to PE21; the same two complexes were detected in the OM-treated sample as in the control. This result is not totally surprising as the previous studies with UV-induction did not detect a different binding pattern with the PE21 sequence and nuclear extracts isolated from UV-irradiated and non irradiated fibroblasts (Noda *et al.*, 2000). Our results combined with the prior study suggest that regulation of p53 transcription through the PE21 element by OM is mediated by mechanisms other than direct alteration of the DNA binding activity of the PE21 interacting proteins. It is possible that there are other cofactors associated with the PE21 binding protein. OM treatment could interfere with this

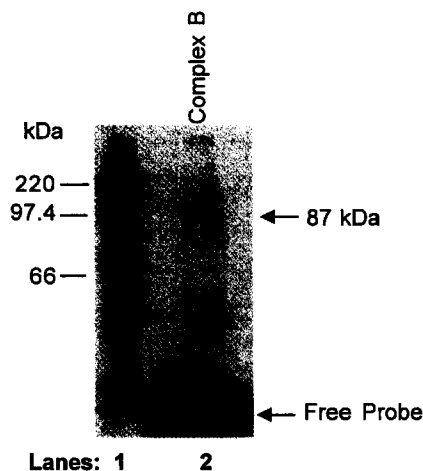


Figure 9 Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked complex B formed with MCF-7 control nuclear extract and the PE21 probe. The positions of 14 C-labeled molecular mass markers are shown in lane 1, and the protein detected from complex B is shown in lane 2. After correction for the bound oligonucleotide, the molecular mass of the band is 87 kDa. A very faint signal, possibly caused by insoluble materials, was seen in the interface of the stacking gel and the separating gel

association. The inability to detect changes of DNA binding activities of transcription factors to a functional regulatory element by gel shift assays has been described in other promoter studies. For example, the transcription of p21^{WAF1/CIP1} gene is activated by TGF β , phobol esters, and histone deacetylase inhibitors through a Sp1 site proximal to the p21 promoter. However, none of these activators altered the DNA binding activity of Sp1 (Datto *et al.*, 1995; Briggs *et al.*, 1996; Nakano *et al.*, 1997; Sowa *et al.*, 1997; Huang *et al.*, 2000).

Previous studies have defined the p53 promoter as a TATA-less and GC-rich less promoter, as the TATA-box and GC-rich sequence are not present in the proximal promoter region of the p53 gene (Tuck and Crawford, 1989). However, our studies with deletion and mutation analysis identified a novel Sp1/Sp3 binding site that covers the regions -174 to -165 of the p53 promoter. This Sp1 binding site is expendable for OM-mediated suppression of p53 transcription but is important for the basal transcriptional activity of p53 gene. Mutation or deletion of this CT-rich sequence decreased p53 promoter activity by 50–60%. The involvement of Sp1/Sp3 in p53 transcription is further demonstrated by our EMSA supershift assay that clearly showed Sp1 and Sp3 binding to this region. Therefore, it is highly likely that Sp1 as a ubiquitously expressed transcription factor has a functional role in p53 gene transcription.

In summary, our studies demonstrate that p53 transcription is down regulated by OM in growth-inhibited and differentiated breast cancer cells. The PE21 element mediates this repressive effect. It is interesting to speculate that the activities of several different intracellular signal transduction pathways converge at the PE21 element. The UV-induced activation of p53 transcription is linked to a stress signal. The OM-induced suppression likely involves the MAP kinase ERK pathway, as we have found that the effects of OM on p53 protein expression and on p53 promoter activity can be partially blocked by the MEK

inhibitor PD98059 (Liu *et al.*, 1999). Further studies to identify and characterize the PE21 element binding proteins will greatly facilitate the understanding of the role of the PE21 site in the control of p53 transcription and its connection to intracellular signaling in normal cells as well as in tumor cells.

Materials and methods

Cells and reagent

The human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Human recombinant OM and LIF were purchased from the R&D systems (Minneapolis, MN, USA).

P53 promoter luciferase reporters

pGL3-p53Luc contains a 599 bp fragment of the human p53 promoter region and exon 1 (-426 to +172) (Liu *et al.*, 1999). To construct 5' Del-1 (-329 to +172), the pGL3-p53Luc was digested with restriction enzyme *Hind*III. The DNA fragment was isolated and subcloned into the *Hind*III site of pGL3-basic vector. Additional 5' and 3' deletion constructs were made by PCR with pGL3-p53Luc as the template. Table 1 describes the primer sequence of oligonucleotides utilized in the deletion and mutation analysis, as well as in EMSAs.

Transient transfection and luciferase assay

MCF-7 cells were plated at a density of 80 000 cells/well in 24-well plates and incubated for 24 h. Cells were cotransfected with 90 ng of various p53 promoter reporter plasmids and 10 ng of a pRL-TK as a normalizing vector per well by using Effectene transfection reagent (Qiagen). Transfected cells were incubated with human recombinant OM at a saturable concentration of 50 ng/ml or the OM dilution buffer (BSA 1 mg/ml in PBS) for 40 h prior to cell lysis. Luciferase activities were measured using the Promega Dual Luciferase Assay System. All the measured fire fly luciferase activity of the plasmid constructs was divided by the renilla

Table 1 Sequences of p53 promoter specific primers. Mutated nucleotides are in boldface and the binding sites are underlined

Primer	Nucleotide sequence 5' to 3'
5' deletion PCR primers	
5' Del-2 5'	GAGCTCAAGCTTCTGCCCTCACAGCTCTGGCTTGCAG
5' Del-3	GAGCTCAAGCTTCACCTCCTCCCAACTCC
5' Del-4	GAGCTCAAGCTTGCTTTTGTGCCAGGAGCCTCG
5' Del-5	GAGCTCAAGCTTGCTCAAGACTGGCGCTAAAGTT
3'	GAAATACGGAGCCGAGAGCC
EMSA oligonucleotides	
P53-Sp1 5'	GCACCTCCTCCCAACTCC
P53-mSp1 5'	GACTCTGCACGCTCGTCGCCAACTCCATTTCTTTGC
P53-PE21 5'	CCTCGCAGGGGTTGATGGGATTGGGGT
P53-mPE21 5'	CCTCGCAGGGGTTGATGAGCTCGGGT
Mutation primers	
P53Luc-mNFkB 5'	GGGGTTGATGGGATTATCGTTTTAAGCTCCCATGTGC
P53Luc-m-c-myc 5'	GGGATGGGGTTTTCCCTCCCTTGGACTCAAGACTGCC
P53Luc-mPE21 5'	GCCTCGCAGGGGTTGATGAGCTCGGGGTTTTCCCTCCG

luciferase activity of pRL-TK to normalize the transfection efficiency.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts of MCF-7 cells were prepared as previously described (Liu *et al.*, 2000). Ten micrograms of nuclear extract were incubated with a ³²P-labeled 27 bp oligonucleotide containing the PE21 sequence for 15 min at RT and loaded onto 6% polyacrylamide gels and run in TGE buffer at 30 mA for 2.5 h at 4°C. The gels were dried and visualized on a PhosphorImager. For UV-cross-linking, after electrophoresis, the wet gel was exposed to a short-wave UV box from a distance of 2–3 cm at 4°C for 1 h as previously described (Phan *et al.*, 1996). Then, the wet gel was briefly exposed to a PhosphorImager screen to locate the complexes. The region of the gel containing complex B was cut out, and the proteins were eluted at room temperature overnight in elution buffer containing 50 mM Tris-HCL (pH 7.9), 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl, and 50 µg/ml gamma-globulin. The eluted protein was precipitated with four volumes of dry ice-cold acetone, washed with ethanol,

and air-dried. After resuspension in Laemmli loading buffer and heating, SDS-polyacrylamide gel electrophoresis was performed and the labeled protein was visualized by a PhosphorImager.

Abbreviations

EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; LIF, leukemia inhibitory factor; OM, oncostatin M; TK, thymidine kinase

Acknowledgments

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References

- Balint E and Reisman D. (1996). *Cancer Research*, **56**, 1648–1653.
- Benoit V, Hellin A, Huygen S, Gielen J, Bours V and Merville M. (2000). *Oncogene*, **19**, 4787–4794.
- Briggs J, Kudlow J and Kraft A. (1996). *J. Biol. Chem.*, **271**, 901–906.
- Datto M, Yu Y and Wang X. (1995). *J. Biol. Chem.*, **270**, 28623–28628.
- Douglas A, Goss A, Sutherland R, Hilton D, Berndt M, Nicola N and Begley C. (1997). *Oncogene*, **14**, 661–669.
- Douglas A, Grant S, Goss G, Clouston D, Sutherland R and Begley C. (1998). *Int. J. Cancer*, **75**, 64–73.
- Estrov Z, Samal B, Kellokumpulehtinen P, Sahin AA, Kurzrock R, Talpaz M and Aggarwal BB. (1995). *J. Interferon Cytokine Res.*, **15**, 905–913.
- Hirose Y, Berger M and Pieper R. (2001). *Cancer Research*, **61**, 1957–1963.
- Horn D, Fitzpatrick W, Gompper P, Ochs V, Bolton-Hanson M, Zarling J, Malik N, Todaro G and Linsley P. (1990). *Growth Factors*, **2**, 157–165.
- Huang L, Sowa Y, Sakai T and Bpardee A. (2000). *Oncogene*, **19**, 5712–5719.
- Ito A, Lai C, Zhao X, Saito S, Hamilton M, Appella E and Yao T. (2001). *EMBO J.*, **20**, 1331–1340.
- Jeffy B, Chen E, Gudas J and Romagnolo D. (2000). *Neoplasia*, **2**, 460–470.
- Kao S, Lemoine F and Marriott S. (2000). *J. Biol. Chem.*, **275**, 35926–35931.
- Kirch H-C, Flaswinkel S, Rumpf H, Brockmann D and Esche H. (1999). *Oncogene*, **18**, 2728–2738.
- Lee M, Song H, Park S and Park J. (1998). *Biol. Chem.*, **379**, 1333–1340.
- Lee M, Song H, Yu S, Lee K and Park J. (1999). *Biochem. Cell. Biol.*, **77**, 209–214.
- Lee S and Rho H. (2000). *Oncogene*, **19**, 468–471.
- Liu J, Spence M, Wallace P, Forcier K, Hellstrom I and Vestal R. (1997). *Cell. Growth Differ.*, **8**, 667–676.
- Liu J, Li C, Ahlborn T, Spence M, Meng L and Boxer L. (1999). *Cell. Growth Differ.*, **5**, 15–18.
- Liu J, Ahlborn T, Briggs M and Kraemer F. (2000). *J. Biol. Chem.*, **275**, 5214–5221.
- Mokdad-Gargouri R, Belhadj K and Gargouri A. (2001). *Nucleic Acids Res.*, **29**, 1222–1227.
- Nakano K, Balint E, Ashcroft M and Vousden K. (2000). *Oncogene*, **19**, 4283–4289.
- Nakano K, Mizuno T, Sowa Y, Orita T, Okuyama Y, Fujita T, Ohtani F, Matsukawa Y and Tokino T. (1997). *J. Biol. Chem.*, **272**, 22199–22206.
- Nayak B and Das B. (1999). *Mol. Biol. Rep.*, **26**, 223–230.
- Noda A, Toma-Aiba Y and Fujiwaba Y. (2000). *Oncogene*, **19**, 21–31.
- Phan SC, Feeley B, Withers D and Boxer LM. (1996). *Mol. Cell. Biol.*, **16**, 2387–2393.
- Peeper D, Dannenberg J, Riele H and Bernards R. (2001). *Nat. Cell. Biol.*, **3**, 198–203.
- Pei X, Nakanish Y, Takayama K, Bai F and Hara N. (1999). *J. Biol. Chem.*, **274**, 35240–35246.
- Raman V, Martensaen S, Reisman D, Evron E, Odenwald W, Faffee M and Sukumar S. (2000). *Nature*, **405**, 974–978.
- Reich N and Levine A. (1984). *Nature*, **308**, 199–201.
- Seluanov A, Gorbunova V, Falcovitz A, Sigal A, Milyavsky M, Zurer I, Shohat G and Goldfinger N. (2001). *Mol. Cell. Biol.*, **21**, 1552–1564.
- Soini Y, Kamel D, Nuorva K, Lane D and Vahakangsa K. (1992). *Pathol. Anat. Histopathol.*, **421**, 415–420.
- Sowa Y, Orita T, Minamikawa S, Nakano K, Mizuno T, Normura H and Sakai T. (1997). *Biochem. Biophys. Res. Comm.*, **241**, 142–150.
- Spence M, Vestal R and Liu J. (1997). *Cancer Research*, **57**, 2223–2228.
- Stuart E, Haffner R, Oren M and Gruss P. (1995). *EMBO J.*, **14**, 5638–5645.
- Tuck S and Caword L. (1989). *Mol. Cell. Biol.*, **9**, 2163–2172.
- Uberti D and Grilli M. (2000). *Amino Acids*, **19**, 253–261.
- Wang J and Friedman E. (2000). *Mol. Carcinog.*, **29**, 179–188.
- Wang X, Ongkeko W, Lau A, Leung K and Poon R. (2001). *Cancer Research*, **61**, 1598–1603.

Xu D, Wang Q, Gruber A, Bjorkholm M, Chen Z, Zaid A, Selivanova G, Peterson C, Wiman K and Pisa P. (2000). *Oncogene*, **19**, 5123–5133.

Zarling J, Shoyab M, Marquardt H, Hanson M, Lionbin M and Todaro G. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 9739–9743.

Zeng X, Keller D, Wu L and Lu H. (2000). *Cancer Research*, **60**, 6184–6188.

Zhu Q, Wani M, El-Mahdy M, Wani G and Wanni A. (2000). *Mol. Carcinog.*, **28**, 215–224.

Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells

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A number of studies have demonstrated that the STAT pathway is an important signaling cascade utilized by the IL-6 cytokine family to regulate a variety of cell functions. However, the downstream target genes of STAT activation that mediate the cytokine-induced cellular responses are largely uncharacterized. The aims of the current study are to determine whether the STAT signaling pathway is critically involved in the oncostatin M (OM)-induced growth inhibition and morphological changes of MCF-7 cells and to identify STAT3-target genes that are utilized by OM to regulate cell growth and morphology. We show that expression of a dominant negative (DN) mutant of STAT3 in MCF-7 cells completely eliminated the antiproliferative activity of OM, whereas expression of DN STAT1 had no effect. The growth inhibition of breast cancer cells was achieved through a concerted action of OM on cell cycle components. We have identified four cell cycle regulators including *c-myc*, cyclin D1, *c/EBP δ* , and p53 as downstream effectors of the OM-activated STAT3 signaling cascade. The expression of these genes is differentially regulated by OM in MCF-7 cells, but is unaffected by OM in MCF-7-dnStat3 stable clones. We also demonstrate that the OM-induced morphological changes are correlated with increased cell motility in a STAT3-dependent manner. Expression analysis of extracellular matrix (ECM) proteins leads to the identification of fibronectin as a novel OM-regulated ECM component. Our studies further reveal that STAT3 plays a key role in the robust induction of fibronectin expression by OM in MCF-7 and T47D cells. These new findings provide a molecular basis for the mechanistic understanding of the effects of OM on cell growth and migration.

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Keywords: oncostatin M; STAT3; cell growth; cell migration; extracellular matrix

Introduction

STAT proteins are important signaling molecules for many cytokines, such as IL-6 family cytokines (Hirano *et al.*, 1997; Heinrich *et al.*, 1998), and numerous growth factors, including EGF (Leaman *et al.*, 1996). STAT proteins possess dual functions that not only transmit a signal from the cell surface to the nucleus after cytokine engagement of cognate cell surface receptors, but also regulate gene expression by direct binding to STAT-recognition sequence in the promoter region of the target genes (Bowman *et al.*, 2000). Although seven members of the STAT family have been characterized in mammalian cells, in general only a single STAT protein or a subset of family members is specifically activated by individual cytokines.

STAT3 and STAT1 are the main STAT proteins activated by the IL-6 cytokine family in a variety of cell types including hepatocytes (Kiuchi *et al.*, 1999; Li *et al.*, 2002; Marsters *et al.*, 2002), chondrocytes (Catterall *et al.*, 2001), astrocytes (Schaefer *et al.*, 2000), endothelial cells (Mahboubi and Pober, 2002), glioblastoma cells (Halfter *et al.*, 2000), melanoma cells (Kortylewski *et al.*, 1999), and breast cancer cells (Badache *et al.*, 2001; Li *et al.*, 2001a, b; Grant *et al.*, 2002). A number of recent studies have shown that activation of STAT3 and STAT1 by the same cytokine, such as oncostatin M (OM), leads to different biological outcomes in different cell types, suggesting that the expression of genetic programs initiated by STAT activation is heavily influenced by cellular context.

OM is a member of IL-6 cytokine family produced by activated T cells and macrophages (Zarling *et al.*, 1986; Brown *et al.*, 1987; Grove *et al.*, 1991). Similar to IL-6 or LIF, OM is pleiotropic and participates in diversified cellular processes such as wound healing (Duncan *et al.*, 1995; Bamber *et al.*, 1998), inflammatory response (Wahl and Wallace, 2001), and cellular proliferation and differentiation (Douglas *et al.*, 1997; Liu *et al.*, 1997; Horn *et al.*, 1990; Grove *et al.*, 1993; Zhang *et al.*, 1994; Halfter *et al.*, 1998). OM manifests its function through specific binding to OM receptors, including the OM-specific receptor (OSMR) and the LIF receptor (LIFR). Additions of OM to cells in culture immediately induce the dimerization of receptor subunits, OSMR β and

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GP130. This results in phosphorylation and activation of receptor-associated JAK family kinases, leading to activation of several intracellular signaling pathways. Although the STAT1 and STAT3 proteins and the MAP kinase ERK are coactivated simultaneously by OM in every cell type that expresses the OM-high-affinity receptor (OSMR), there are conflicting reports as to which signaling cascade is critically linked to a defined OM-induced cellular functional change.

Previously, we have shown that the OM-induced growth suppression and morphological changes of breast cancer cell line MDA-MB231 can be totally abrogated by blocking ERK activation with the MAP kinase kinase-1 (MEK-1) inhibitors (Li *et al.*, 2001a,b,c). By contrast, MEK inhibitors PD98059 and U0126 were not able to abolish the OM antiproliferative activity or to reverse the morphological changes in MCF-7 cells, implying that other signaling pathways activated by OM in MCF-7 cells are responsible for its actions.

It has been reported in several studies that the OM antiproliferative activity is accompanied by the induction of morphological changes of breast cancer cells (Liu *et al.*, 1997; Spence *et al.*, 1997; Douglas *et al.*, 1998; Halfter *et al.*, 1998). In general, the OM-treated cells displayed disrupted intercellular cell junctions. Cells became scattered. The morphological changes could be partially attributed to cellular differentiation, as the accumulation of neutral lipid, a marker of differentiation, was detected in OM-treated MCF-7 cells (Douglas *et al.*, 1998; Grant *et al.*, 2002). However, a recent study conducted in T47D cells has suggested that the scattered phenotype is associated with an increased cell migration towards OM (Badache and Hynes, 2001). OM, acted as a chemoattractant, induced T47D cells to migrate in the absence of STAT3 activation. The mechanisms that underlie the effect of OM on cell migration of T47D cells remain elusive.

The aims of the current study are to determine whether the STAT signaling pathway is critically involved in the OM-induced growth inhibition and morphological changes of MCF-7 cells and to identify STAT3-target genes that are utilized by OM to regulate cell growth and motility.

Result

Blockade of OM-induced STAT3 and STAT1 transactivation by dominant negative STAT mutant proteins

OM activates both STAT3 and STAT1 in MCF-7 cells. To determine whether STAT3 or STAT1 activation is a key event in the OM-induced growth inhibition of MCF-7 cells, we established stable MCF-7 clones that express a dominant negative STAT3 mutant (dnStat3, Y705F) or a dominant negative STAT1 mutant (dnStat1, Y701F). MCF-7 clones (neo) transfected with the empty vector (pEFneo) were also generated and were used in this study as negative controls to access

the possible side effects associated with antibiotic selection.

To determine the effect of mutant STAT proteins on OM-induced STAT DNA binding activity, gel shift and supershift assays using a ³²P-labeled oligonucleotide probe (c-FosSIE), containing the high-affinity STAT3 binding site of c-fos gene promoter, were performed with nuclear extracts prepared from MCF-7 stable clones that were untreated or treated with OM for 15 min. As shown in Figure 1a, in MCF-7-neo cells, OM induced the formation of three specific DNA-protein complexes (lane 2). Supershift assays with antibodies specific to STAT1 or to STAT3 showed that the complex C3 was completely supershifted by anti-STAT1 antibody (lane 3), suggesting that C3 is the homodimer of STAT1. The C2 complex was supershifted by both anti-STAT1 and anti-STAT3 (lane 5), thereby demonstrating that C2 is the heterodimer of STAT1 and STAT3. The low-intensity band C1 was completely supershifted by anti-STAT3 antibody (lane 4), demonstrating its identity as the STAT3 homodimer. The OM-induced STAT binding activity was markedly reduced in clones of dnStat3 (lanes 8–11) and dnStat1 (lanes 12–15) as compared to the neo clone (lanes 1–7) and untransfected MCF-7 cells (data not shown). Low levels of STAT1 binding activity were observed in uninduced neo (lane 1) and dnStat3 (lane 8) clones, suggesting that some of the STAT1 protein exist in a constitutively activated form in MCF-7 cells.

To further demonstrate a blockade of STAT3 transactivating activity by the mutant dnStat3 a STAT3 luciferase reporter (pTKlucS3) was transiently transfected into MCF-7-neo and dnStat3 clones. Cells, 40 h after transfection, were treated with OM for 4 h and luciferase activities were measured. As shown in Figure 1b, OM induced an eight-fold increase in the promoter activity of pTKlucS3 in the neo clone, but this induction was completely abolished in the clone of dnStat3.

We next examined the effect of OM on ERK activation in MCF-7 and stable clones. Western blot analysis detected comparable levels of activated ERK in parental MCF-7 cells and stable clones (Figure 2). These results clearly demonstrate that expression of the DN STATs specifically abolished STAT DNA binding and transactivating activity without subverting the OM-induced MEK/ERK signaling pathway.

Expression of dnStat3 but not dnStat1 abolished the antiproliferative activity of OM

The impact of dnStat3 or dnStat1 expression on OM-induced growth suppression was first evaluated by cell proliferation assays that measured the binding of a fluorescent dye to cellular nucleic acids, which produces fluorescent signals in proportion to the cell number. Figure 3a shows that the cellular proliferation of MCF-7, the neo clones, and the clones expressing dnStat1 was inhibited by 60–75% as compared to control after incubation with OM for 5 days, whereas the growth rate of dnStat3 clones was unaffected by OM. To further

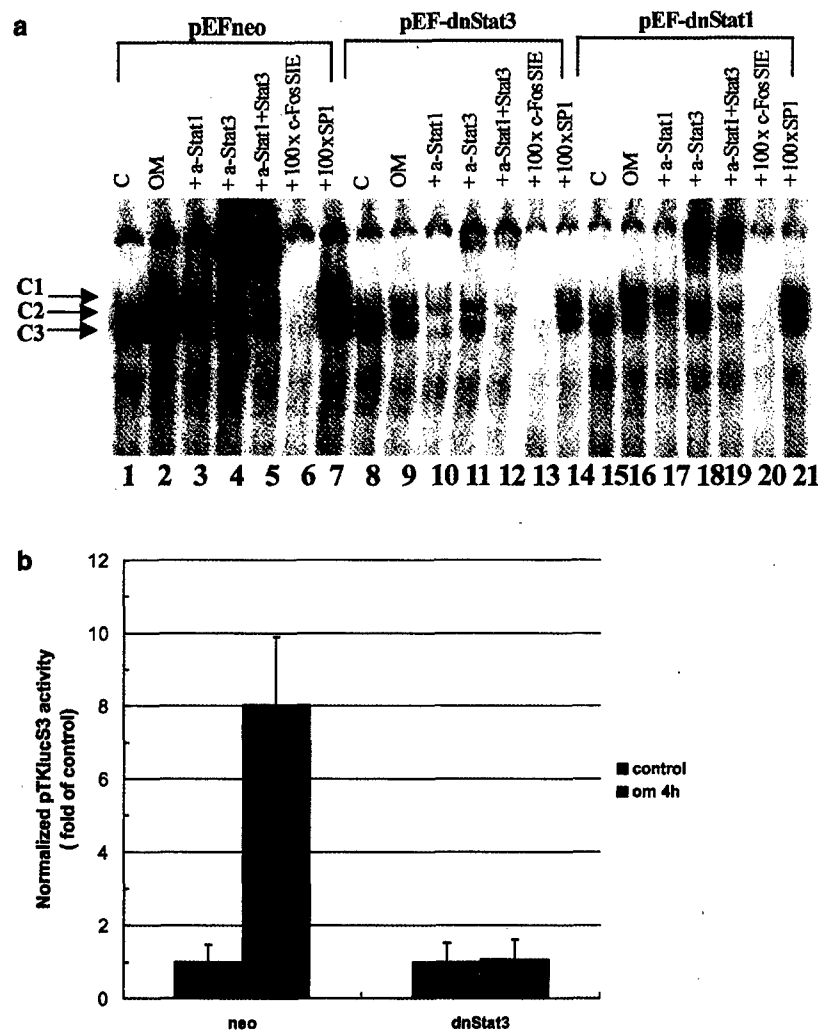


Figure 1 Blocking STAT DNA binding and transactivation by STAT mutant proteins. (a) EMSA analyses of nuclear proteins interacting with the STAT binding site. Nuclear extracts were prepared from MCF-7-neo clone, pEFneo-dnStat1 clone, and pEF-dnStat3 clone that were untreated (lanes 1), or treated with OM (15 min) (lanes 2–15). A double-stranded oligonucleotide, designated as c-FosSIE, was radiolabeled and incubated with 10 μ g of nuclear extract per reaction for 10 min at 22°C in the absence (lanes 1, 2, 7, 8, 14, 15) or presence of 100-fold molar amounts of unlabeled competitor DNA (lanes 6, 7, 13, 14, 20, 21). For supershift, antibodies were incubated with nuclear extracts at 22°C for 30 min prior to the addition of the probe. The reaction mixtures were loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 3 h at 4°C. (b) Analysis of STAT3 reporter luciferase activity. The STAT3 reporter pLucTKS3 was cotransfected with pRL-SV40 into neo or dnStat3 clones. Cells, 40 h after transfection, were treated either with OM (50 ng/ml) or with OM dilution buffer for 4 h prior to harvesting cell lysates. Luciferase activities in total cell lysates were measured using the Promega Dual Luciferase Assay System. Absolute firefly luciferase activity was normalized against renilla luciferase activity to correct for transfection efficiency. The normalized luciferase activity is expressed as the fold of luciferase activity in untreated control cells. The data presented are derived from three separate transfections in which triplicate wells were used in each condition.

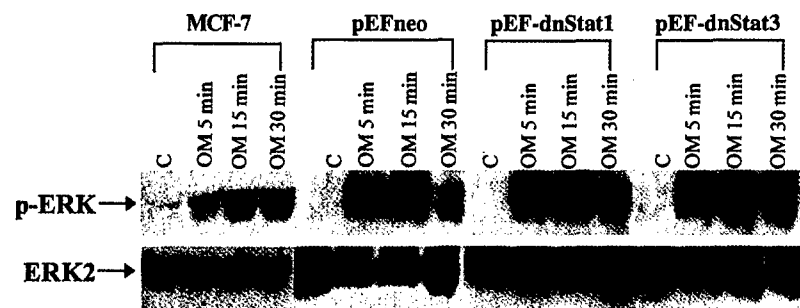


Figure 2 Activation of MAP kinases ERK1 and ERK2 by OM in MCF-7 and stable clones. MCF-7, neo, dnStat1, and dnStat3 clones cultured in medium containing 0.5% FBS were stimulated with 50 ng/ml OM. At the indicated times, cells were scraped into lysis buffer and cell extracts were prepared. Soluble proteins (30 μ g/lane) were applied to SDS-PAGE. Detection of phosphorylated ERK1 and ERK2 and the nonphosphorylated ERK2 was performed by immunoblotting.

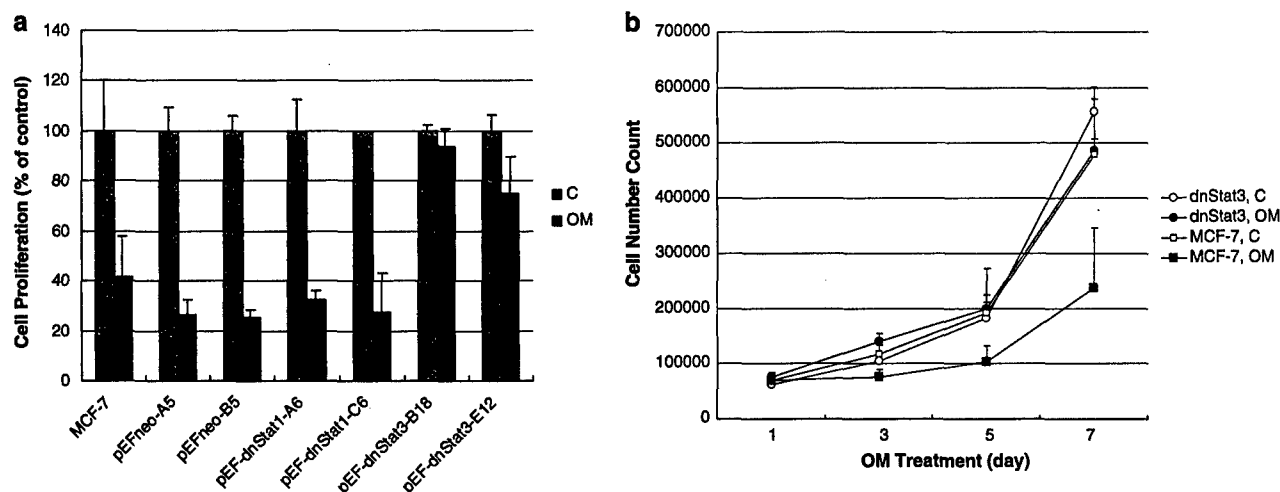


Figure 3 Abrogation of OM antiproliferative activity by expression of DN STAT3 in MCF-7 cells. (a) Untransfected parental MCF-7, two independently isolated neo, dnStat1, and dnStat3 clones were cultured in 96-well plates at a density of 2000 cells/well in 0.1 ml RPMI containing 2% FBS with or without 50 ng/ml OM for 5 days. The medium was removed and cells were washed with PBS. A volume of 200 μ l of Cyquant GR dye mixed with cell lysis buffer was added to each well. The fluorescent signals were then measured using a fluorescence microplate reader. (b) Cells of MCF-7 and dnStat3 clone were cultured in 24-well culture plates at a density of 7×10^3 cells/well in 0.5 ml RPMI containing 2% FBS with or without 50 ng/ml of OM for different days. At the indicated time, cells were trypsinized and viable cells (trypan blue excluding cells) were counted. Values are mean of triplicate wells. The figure shown is representative of 4–5 separate experiments

verify the blocking effect of DN STAT3 on OM growth inhibitory activity, a time course of cell growth rate in the absence or the presence of OM was conducted by direct accounting of the viable cell numbers of MCF-7 cells and the dnStat3 clone. Figure 3b shows that OM exerted a time-dependent inhibitory effect on MCF-7 cells. By 7 days of the OM treatment, the number of viable cells was decreased by more than 50% as compared to control. Consistent with the results of Figure 3a, the growth of dnStat3 cells was not inhibited by OM through the entire duration of the experiment. These results demonstrate that expression of the dominant negative mutant of STAT3 but not STAT1 blocked the OM-mediated growth arrest in MCF-7 cells.

OM regulates *c-myc* gene expression through STAT3-dependent and independent mechanisms

c-myc is a potent oncogene, the expression level of which is directly correlated with cellular growth status (Kelly et al., 1983; Carroll et al., 2002). Recent studies further identify *c-myc* as a target gene of STAT3 (Kiuchi et al., 1999; Bowman et al., 2001). OM and IL-6 regulates *c-myc* mRNA expression in a biphasic manner with an early induction and a subsequent suppression (Liu et al., 1992, 1997; Minami et al., 1996; Spence et al., 1997). To determine the role of STAT3 in OM-regulated transcription of *c-myc*, Northern blot analysis was conducted to detect levels of the *c-myc* mRNA after short and long exposures to OM in MCF-7 and stable clones. Figure 4a shows that OM treatment over a 3-day time course decreased the levels of *c-myc* mRNA by 60–80% in MCF-7, neo, and dnStat1 clones but not in dnStat3 clones. Interest-

ingly, contrary to the long exposure, the transient induction of *c-myc* mRNA by OM was not abolished by overexpression of dnStat3 or dnStat1 (Figure 4b). A brief incubation of cells with OM stimulated *c-myc* expression to comparable levels (2–4 fold) in the MCF-7, neo, dnStat1, and the dnStat3 clones. These results suggest that OM downregulates *c-myc* transcription through a STAT3-dependent mechanism, whereas the immediate effect of OM on upregulation of *c-myc* is independent of the STAT3 signaling cascade.

Identification of STAT3 target genes *c/EBP δ* and cyclin D1 as novel OM-regulated genes that participate in OM-mediated growth repression

Previous investigations have shown that OM treatment resulted in an accumulation of breast cancer cells in the G₀/G₁ phase of the cell cycle (Douglas et al., 1998; Grant et al., 2002). The molecular mechanisms underlying the OM effects on cell cycle have not been clearly defined. Since cyclin D1 (Sinibaldi et al., 2000; Sauter et al., 2002) and *c/EBP δ* (Yamada et al., 1997) are known target genes of STAT3 activation, and their gene products are important regulators in the G₀/G₁ phase of the cell cycle, we sought to determine whether OM regulates cyclin D1 and *c/EBP δ* in MCF-7 cells and whether this regulation requires STAT3 activity. Figure 5 shows that OM reciprocally modulates cyclin D1 and *c/EBP δ* expression. OM increased *c/EBP δ* protein expression to levels of three- to fivefold of control in MCF-7, the neo, and dnStat1 clones, whereas the *c/EBP δ* expression in dnStat3 cells was not induced by OM (Figure 5a). The expression of cyclin D1 was inhibited by OM in MCF-7 cells and this inhibition was

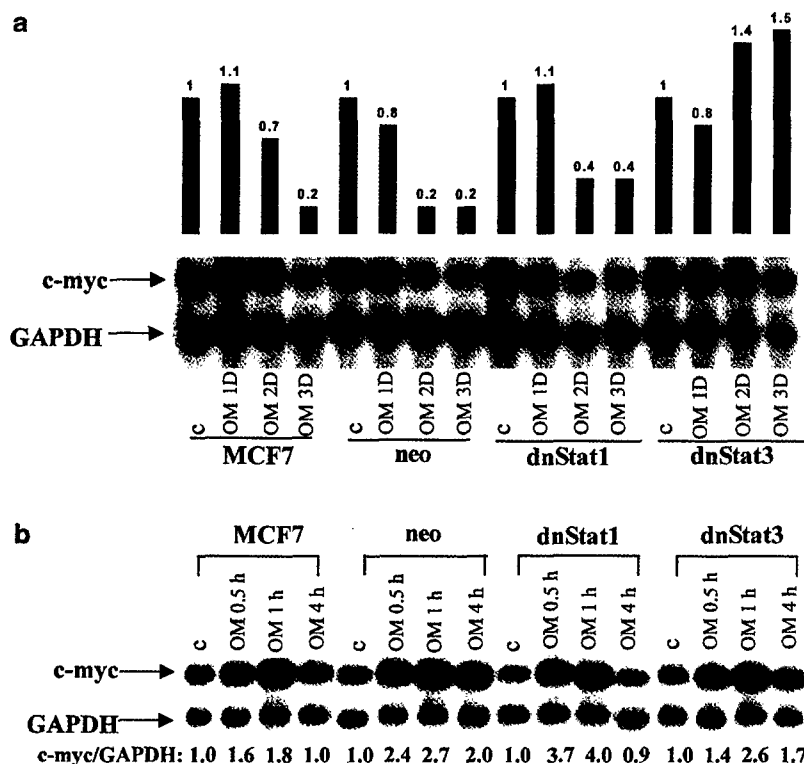


Figure 4 Detection of *c-myc* mRNA expression in MCF-7, neo, dnStat1 and dnStat3 clones by Northern blot analysis. Cells cultured in 60 mm dishes were treated with OM for 1–3 days (a) or for a short period of time (b). By the end of treatment, cells were lysed and total RNA was isolated. Total RNA of 15 μ g per sample was analysed for *c-myc* mRNA by Northern blot. The membrane was stripped and rehybridized to a human GAPDH cDNA probe. The figure shown is representative of three separate experiments

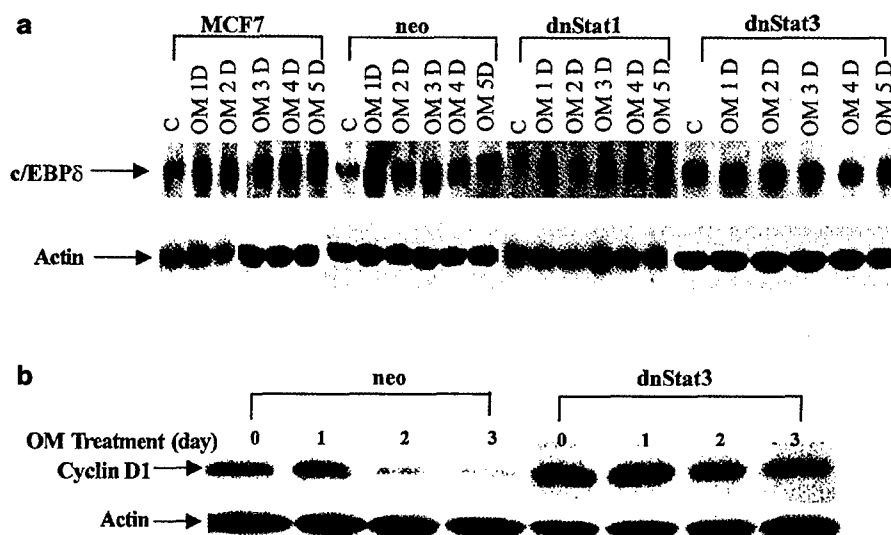


Figure 5 Western blot analyses of *c/EBPδ* and cyclin D1 protein expressions in MCF-7 and stable clones. Cells cultured in medium containing 2% FBS were incubated with 50 ng/ml OM for the indicated times and total cell lysate was harvested at the end of treatment. Soluble proteins (50 μ g/lane) were applied to SDS-PAGE. Detections of *c/EBPδ* (a) and cyclin D1 (b) were performed by immunoblotting and autoradiography. Immunoblotting of the membranes with anti- β -actin mAb was conducted to normalize the amounts of protein being analysed

abrogated by overexpression of dnStat3 (Figure 5b). These results demonstrate that OM exerts its effect on cell growth by direct regulation of critical cell cycle components through the STAT3 signaling pathway.

STAT3 participates in OM-mediated downregulation of p53

OM downregulates p53 expression in MCF-7 cells by inhibiting the gene transcription (Liu *et al.*, 1999; Li *et al.*,

2001a,b,c). Since blocking the MEK/ERK pathway only partially reversed the OM inhibitory effect on p53 protein expression, it is possible that other signaling pathways could also be involved. To evaluate the role of STAT3 in p53 transcription, a p53 promoter luciferase reporter construct pGL3-p53 was cotransfected with pEF-dnStat3 or with a control vector (pEFneo) into MCF-7 cells along with pRL-SV40 for normalization of variations in transfection efficiency. Cells were treated with OM or OM dilution buffer for 40 h and dual luciferase activities were measured in total cell lysates. The p53 promoter activity was decreased by 50% in OM-treated cells in the absence of pEF-dnStat3. Expression of dnStat3 reversed the OM inhibitory effect on p53 promoter activity (Figure 6a). We further examined p53 protein levels in MCF-7 neo and dnStat3 clones untreated or treated with OM. Western blot analysis shows that while OM treatment lowered p53 protein level to 35% of control in the neo clone, the level of p53 protein in the dnStat3 clone was not decreased by OM treatment (Figure 6b). Taken together, these results demonstrate that activation of STAT3 signaling pathway is a necessary step in the OM-mediated regulation of p53 transcription.

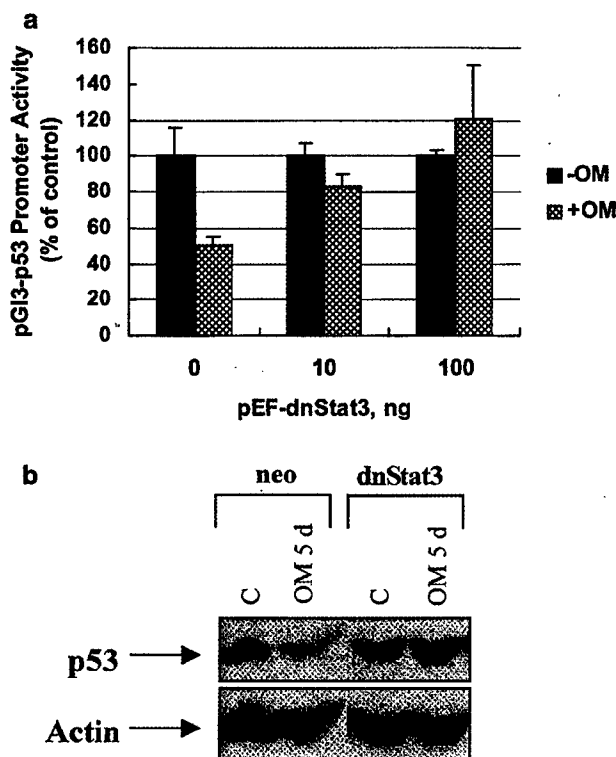


Figure 6 Evaluation of the role of STAT3 in OM-mediated downregulation of p53 promoter activity and protein expression. (a) The p53 promoter reporter pGL3-p53 was cotransfected with pEF-dnStat3 or with pEFneo into cells along with the normalizing vector pRL-SV40. Transfected cells were treated either with OM (50 ng/ml) or with OM dilution buffer for 40 h prior to harvesting cell lysates. The normalized luciferase activity is expressed as the percentage of luciferase activity in untreated control cells. (b) Cells were cultured in the presence or absence of OM for 5 days and harvested. Western blot analysis of p53 protein expression was conducted using total cell lysates

OM-induced morphological changes are associated with increased cell motility and expression of fibronectin in a STAT3-dependent manner

Figure 7 shows OM-induced morphological changes that appeared in MCF-7, neo clone, and dnStat1 clone, but not in dnStat3 clones. The tight cell-to-cell junctions in MCF-7 cells was severely disrupted by OM. Cells became flat and larger, and also developed cell extensions and membrane protrusions. These changes subtly surfaced after 1 day of OM treatment and were predominant after 3 days. However, in dnStat3 clones morphological changes were not readily detected even after 5 days. After a longer period of culture (7 days) in the presence of OM, slight morphological changes were noticed. The OM-induced morphological changes were reversible, as cells slowly resumed original cell shape after withdrawal of OM from the culture medium.

We were interested to know whether the OM-induced phenotype is related to changes in the extracellular matrix (ECM) composition. Using Western blot analysis, we examined several ECM proteins including P-cadherin, E-cadherin, and fibronectin that are known to play important roles in cell morphology and migration. We found that the protein level of E-cadherin was not changed by OM and the level of P-cadherin was only slightly increased after OM treatment (data not shown). In contrast, OM induced a robust time-dependent expression of fibronectin in MCF-7 cells and a much weak induction of fibronectin in the dnStat3 clone (Figure 8a). To determine whether the effect of OM on fibronectin expression is confined to MCF-7 cells, we examined fibronectin expression in another two breast cancer cell lines T47D and SKBR-3 to which OM also induces similar morphological changes (data not shown). Figure 8b shows that the expression of fibronectin in these two cell lines were strongly induced by OM as well. Moreover, the induction of fibronectin by OM was not detected in T47D-dnStat3 cells that stably express the dnStat3 mutant (Figure 8c) and these cells failed to show the distinct morphological changes upon OM treatment. These results strongly suggest that fibronectin is a downstream effector of the OM-activated STAT3 signaling cascade and expression of fibronectin may change the cell morphology.

Finally, we examined the involvement of STAT3 pathway in cell migration. Boyden chamber assays were performed to examine the direct effect of OM on cell motility. Cells were pretreated with OM for different days, trypsinized, counted, and seeded onto the top chamber; cells were then allowed to migrate through the membrane in the absence of any chemoattractant. As shown in Figure 9a, OM induced a time-dependent increase in the number of migrated cells. After a 2-day treatment, the number of migrated cells increased more than 12-fold of control. To determine the involvement of STAT3 in this newly discovered property of OM, the migration assay was conducted using DN STAT3 cells that were untreated or treated

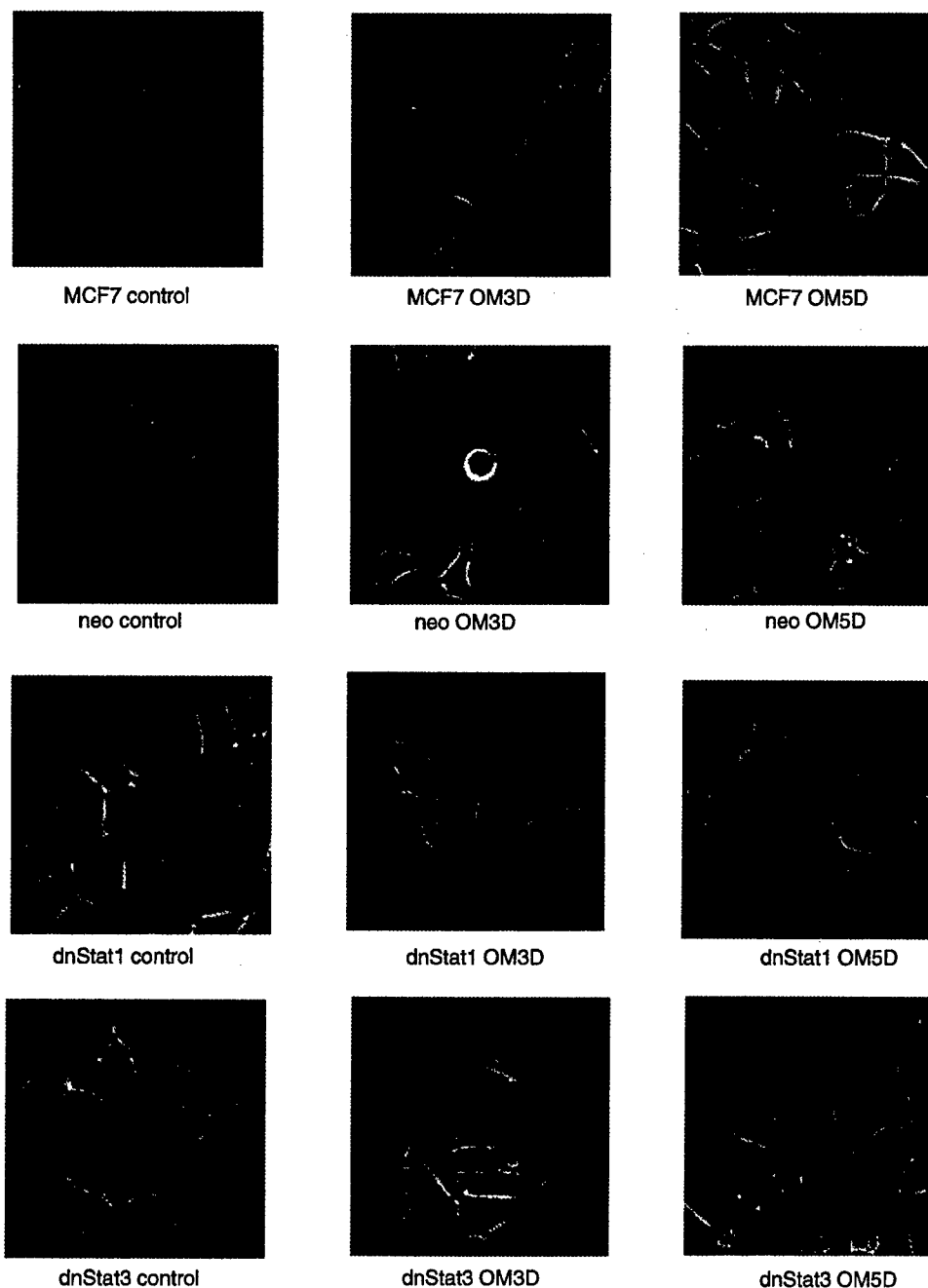


Figure 7 Detection of OM-induced morphological changes in MCF-7, neo, and dnStat1 clones but not in the dnStat3 clone. Cells were cultured in medium containing 2% FBS with or without OM for 3–5 days. Photographs were taken at the indicated time of OM treatment by using the Penguin 600CL digital camera at a magnification of 200. The loss of OM-induced morphological changes in T47D-dnStat3 cells was also observed

with OM. Figure 9b shows that the cell motility of dnStat3 clones was not stimulated at all even after 6 days of OM treatment.

Previously, using OM as a chemoattractant added to the bottom chamber, Badache *et al* has shown that expression of DN STAT3 did not affect the migration of T47D cells (Badache and Hynes, 2001). To determine whether DN STAT3 expression in MCF-7 cells is able to block the cell migration towards OM, MCF-7 and the dnStat3 clone were directly seeded onto the top

chambers without prior exposure to OM. OM or its dilution buffer as control was added to the bottom chambers. Cells were allowed to migrate for 24 h and the migrated cells were stained and counted. Figure 9c shows that OM as a chemoattractant induced the migration of dnStat3 clone and MCF-7 to similar extents. This corroborated the observation made in T47D cells. Thus, our results, for the first time, demonstrate that OM affects the intrinsic cell motility through a STAT3-dependent mechanism, whereas the

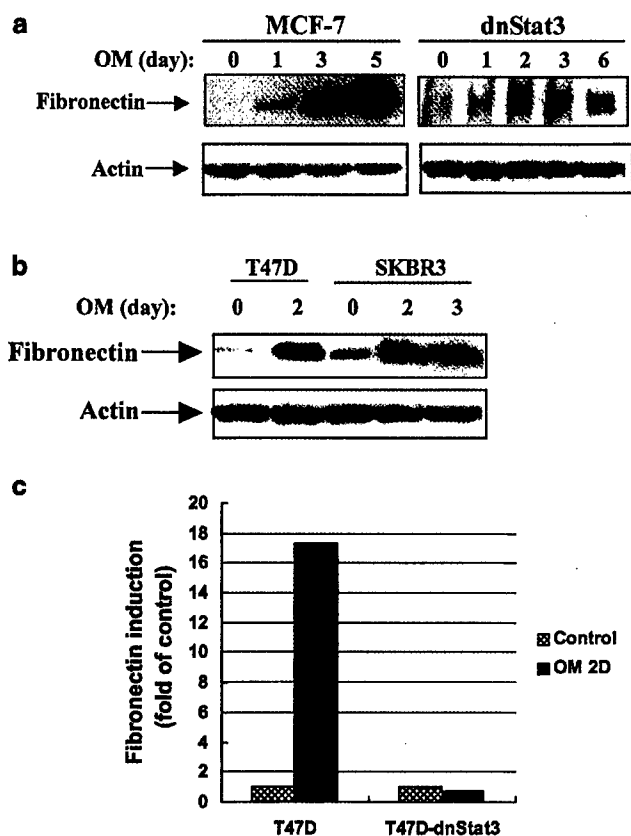


Figure 8 Western blot analysis for fibronectin expression. The effects of OM on fibronectin expression were examined in wild-type STAT3 and the dnStat3 clones. MCF-7, MCF7-dnStat3 (a), T47D, SKBR-3 (b) cells were treated with OM. At the indicated time, cell was harvested and total cell lysates of 50 μ g per sample were used to analyse fibronectin expression by Western blot analysis. In (c), T47D and T47D-dnStat3 cells were cultured in the absence (control) or presence of OM for 4 days and the expression of fibronectin was examined by Western blot using total cell lysate. Data are expressed as fold increase relative to control

mechanism of chemoattraction mediated by OM is independent of STAT signaling pathway.

Discussion

The STAT pathway and the MEK/ERK pathway are two major signaling cascades utilized by IL-6 cytokine family to elicit a variety of biological response (Heinrich *et al.*, 1998). Depending on cell types, activation of the same pathway can lead to different biological outcomes. It is conceivable that the downstream targets of ERK or STAT are differentially activated in different cell lines. Each signaling pathway may regulate a unique set of genes whose functions dictate the outcome induced by the cytokine in a cell line-specific manner. From this point of view, identification of target genes of a specific signaling cascade is of importance. It can provide insight to understand the mechanisms of cytokine's action at the molecular levels and may help predict outcomes in an uncharacterized system.

In this study, by utilizing dominant negative mutants of STAT3 and STAT1 we demonstrate that STAT3 but not STAT1 activation is a critical event in the OM-mediated growth inhibition of MCF-7 cells. Our results are consistent with the finding in A375 melanoma cells whose growth was strongly inhibited by OM in a STAT3 but not a STAT1-dependent mechanism (Kortylewski *et al.*, 1999). While it has been shown that STAT1 activation by IL-4 results in reduced growth rates in human colon carcinoma cell lines (Chang *et al.*, 2000), it appears that activation of Stat1 by OM alone does not lead to changes in gene transcription and cell function. However, since blocking STAT3 DNA binding activity could affect the binding activity of STAT1 protein, our data cannot absolutely rule out the functional role of STAT1 in OM signaling. Additional experiments to block STAT3 and STAT1 expression by different approaches will be needed to reach a firm conclusion.

Recent investigations have identified *c-myc* as the downstream effector of STAT3 signaling (Kiuchi *et al.*, 1999; Bowman *et al.*, 2001). By using the dnStat3 clone, we found that the OM-induced biphasic regulation of *c-myc* is both STAT3-dependent and STAT3-independent. Our finding that dnStat3 blocks the OM-induced suppression of *c-myc* transcription recapitulates the observation obtained previously in M1 cells (Minami *et al.*, 1996). It was shown that IL-6-induced down-regulation of *c-myc* in M1 cells was obviated by dnStat3 expression. Unexpectedly, the rapid induction of *c-myc* mRNA expression by OM in MCF-7 cells was not affected by dnStat3. The induction of *c-myc* mRNA (three- to four fold of control) by OM was completely abolished by actinomycin D (data not shown), implying a nature of transcriptional activation. The E2F binding site of the *c-myc* promoter, located at +98 to +106 bp, was shown to interact with STAT3 and to mediate the inducing activity of IL-6 on *c-myc* promoter activity (Kiuchi *et al.*, 1999). We have analysed a series of *c-myc* promoter luciferase reporters that contain the wild-type E2F or the mutated E2F sites in a transient transfection system of MCF-7 cells. We did not observe a significant induction of the *c-myc* promoter activity by OM regardless of the status of the E2F site (our unpublished data). Our results suggest that OM may stimulate *c-myc* transcription through some regulatory mechanisms such as chromatin remodeling that might not be readily accessed by the transient transfection of plasmid DNA. The mechanisms underlying the STAT3-independent activation of *c-myc* transcription by OM is currently under investigation in our laboratory.

In addition to *c-myc*, we have identified another two cell cycle regulators, *c/EBP δ* and cyclin D1, that function in the G₀/G₁ phase of the cell cycle. *c/EBP δ* has an important role in the induction of G₀ growth arrest in mammary epithelial cells (Hutt *et al.*, 2000) and cyclin D1 expression promotes cell cycle progression (Sherr and Roberts, 1999). OM through the STAT3 signaling cascade coordinately regulates the expression of *c/EBP δ* and cyclin D1. Although the direct role of these proteins individually in the OM-mediated growth arrest of MCF-7 cells has not been demonstrated in this

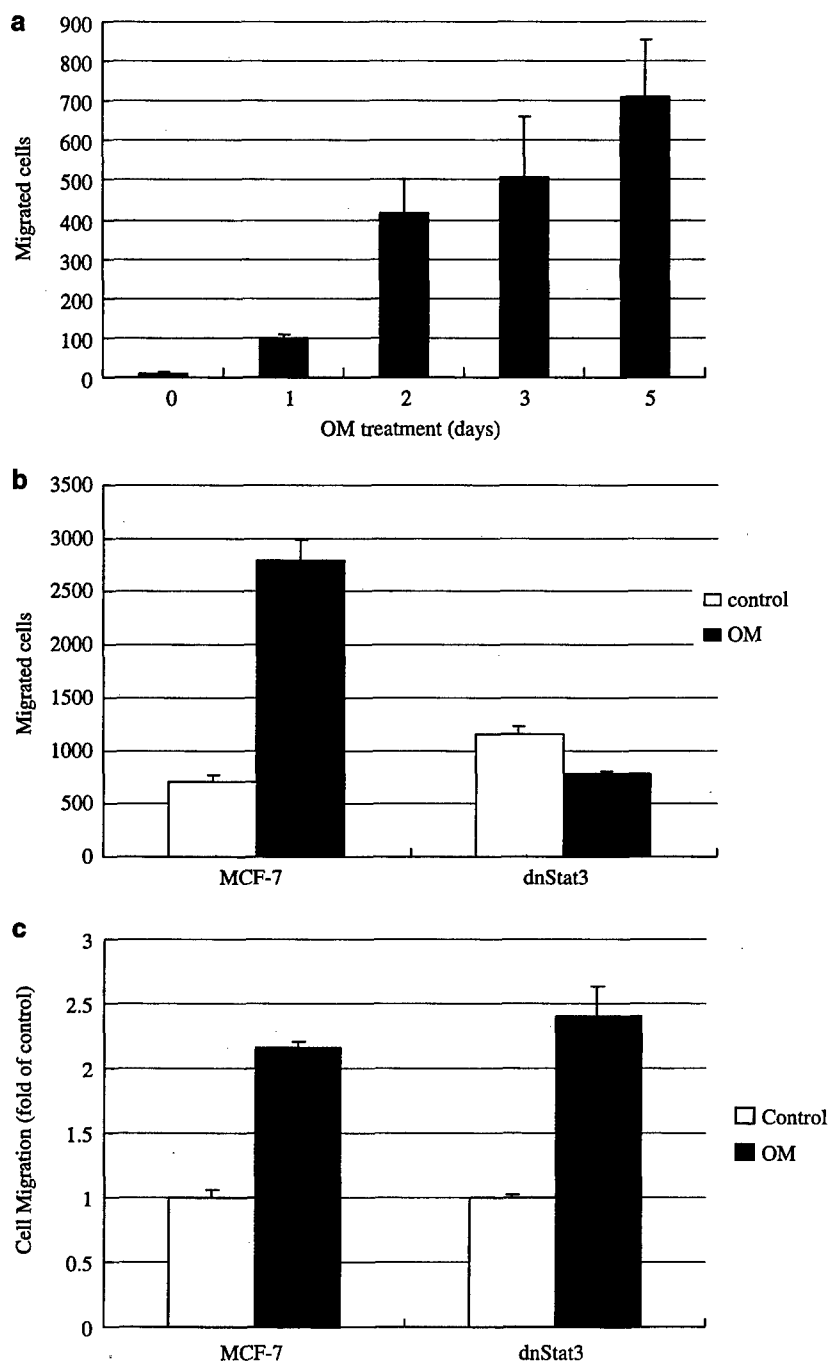


Figure 9 OM increases the intrinsic cell motility through the STAT3-signaling cascade. The effects of OM on cell motility were examined in MCF-7 and the dnStat3 clone. In (a), MCF-7 cells were pretreated with OM for different days at a concentration of 50 ng/ml. At the end of treatment, cells were trypsinized and counted. Cells were then seeded onto the top chamber at a density of 0.15×10^6 per chamber. The top chamber and the bottom chamber both contained 2% FBS RPMI. Migrated cells were counted after 6 h. In (b), MCF-7 and MCF7-dnStat3 cells were cultured in the absence (control) or presence of OM for 6 days. The ability of cell to migrate was determined as described in (a). In (c), cells without prior exposure to OM were seeded onto the top chamber which contained 2% FBS RPMI. OM at a concentration of 50 ng/ml was added to the bottom chamber that contained 2% FBS RPMI. Cells were allowed to migrate towards OM for 24 h. The migrated cells were fixed, stained, and counted. Data are expressed as fold increase relative to control

study, we postulate that the reduced growth rate of MCF-7 cells results from a concerted regulatory action of OM on several cell cycle components, leading to an accumulation of cells at the G_0/G_1 phase.

Recently, there is emerging information to link STAT3 signaling pathway with p53. It was shown that the expression of the wild-type p53 in breast cancer cells inhibited STAT3-dependent transcriptional activity

(Lin *et al.*, 2002). Another reporter showed that Hep3B cells stably expressing a temperature-sensitive p53 species (p53-Val-135) displayed a reduced response to IL-6 when cultured at the wild-type p53 permitting temperature (Rayanade *et al.*, 1997). Later studies revealed that the reduction of cellular response to IL-6 was because of a p53-caused masking of STAT3 and STAT5, but not STAT1 (Rayanade *et al.*, 1998). In this study, we showed that blocking STAT3 activity by dnStat3 reversed the OM inhibitory effect on p53 transcription, demonstrating an involvement of STAT3 in OM-mediated negative regulation of the p53 transcription. Our previous investigation has identified the regulatory sequence (PE21) of p53 promoter as the OM-responsive element that mediates the OM effect on p53 transcription (Li *et al.*, 2001a, b, c). However, we did not detect STAT3 in the protein complex bound to the PE21 region by supershift using anti-STAT3 antibody (our unpublished data). This is not surprising, as the sequence of PE21 motif is not related to the STAT canonical sequence (Noda *et al.*, 2000). We speculate that the effect of STAT3 on p53 transcription is likely indirect and might be mediated through other downstream effectors of STAT3. Nevertheless, our novel finding that STAT3 participates in p53 transcription brings new insight into the interaction between the STAT signaling machinery and p53. It is possible that a reciprocal interaction exists: p53 regulates STAT3 phosphorylation and transactivating activity and the STAT3 affects p53 function by controlling p53 transcription.

OM has been implicated in the process of wound healing, which involves cell proliferation, migration, and remodeling of ECM. In dermal fibroblasts, OM stimulates the production of ECM components such as collagen and glycosaminoglycan (Duncan *et al.*, 1995). OM has been reported to stimulate the synthesis of tissue inhibitor of metalloproteinases 1 and 3 (Kerr *et al.*, 1999; Li *et al.*, 2001a, b, c). In endothelial cells, OM-promoted cell migration is associated with induction of the urokinase plasminogen activator (uPA) and uPA receptor (Strand *et al.*, 2000). In this study, we provide the first evidence that OM strongly induces fibronectin protein production. Analysis of fibronectin mRNA in untreated and OM-treated cells showed that OM increased the levels of fibronectin mRNA to the order of 20–30-fold (data not shown). The induction at this order of magnitude is likely to be transcriptional. Fibronectin is a multifunctional adhesive glycoprotein (Makogonenko *et al.*, 2002). It affects cell adhesion and migration. The OM-induced cell migration is likely to be mediated through the interaction of fibronectin with other ECM components. In MCF-7 cells, the OM-induced morphological changes, increased intrinsic cell motility, and production of fibronectin are all inhibited by DN STAT3. Our studies suggest that STAT3 signaling cascade may play important roles in ECM remodeling in breast cancer cells.

In summary, our studies have defined c-myc, cyclin D1, c/EBP δ , and p53 as the downstream effectors of the OM-activated STAT3 signaling cascade that participate in the process of growth regulation. Furthermore, we

have unraveled the importance of STAT3 activation in OM-induced migration of breast cancer cells and identified a new OM-regulated ECM component fibronectin. These results provide a better understanding of the molecular mechanisms whereby OM regulates cell growth and differentiation.

Materials and methods

Cells and reagents

Human breast cancer cell lines MCF-7, T47D, and SKBR-3 were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The T47D-dnStat3 cells were kindly provided by Dr Ali Badache at Friedrich Miescher Institute, Basel, Switzerland, and the effects of OM on these cells have been described (Badache and Hynes, 2001). The plasmids pEFneo and pEFneo-dnStat1 (Y701F) (Chang *et al.*, 2000) were obtained from Dr Xin-Yuan Fu at Yale University. The plasmid pEF-dnStat3 (Y705F) (Minami *et al.*, 1996) was provided by Dr Shizuo Arika at Osaka University. The specific STAT3 luciferase reporter plasmid pLucTKS3 (Zhang *et al.*, 1996) and the control reporter pLucTK were provided by Dr Richard Jove at the University of South Florida College of Medicine, Tampa, FL, USA. Antibodies directly to STAT3, STAT1, ERK2, cyclin D1, c/EBP δ , p53, c-myc, and fibronectin were obtained from Santa Cruz and the antiphosphorylated ERK was obtained from Cell Signaling Technology.

Generation of stable clones for STAT3 and STAT1 mutant proteins

To generate stable MCF-7 clones that constitutively express an FLAG-tagged dominant negative (DN) form of STAT3 (Y705F), plasmid pEF-dnStat3 and a empty vector containing a neomycin-resistant gene (pEFneo) were cotransfected into MCF-7 cells using the transfection reagent Effectene (Qiagen, Valencia, CA, USA). Cells were selected in 300 μ g/ml G418. Several clones were picked, expanded in the presence of G418, and analysed for dnSTAT3 expression with anti-FLAG antibody by immunostaining. To generate MCF-7 clones expressing a DN form of STAT1 (Y701F), plasmid pEFneo-dnStat1 was transfected into MCF-7 cells and several independent clones were selected and characterized for dnStat1 expression by Western blot. MCF-7-neo clones were established by introducing the empty vector pEFneo into MCF-7 cells. These clones were used as negative controls in this study.

For each stable cell lines, at least two independent clones were analysed by Western blot analysis for the expression of mutant STAT proteins, by gel shift for STAT DNA binding activity, and by growth assays to determine the response to OM treatment. Significant clonal variations were not observed.

Electrophoresis mobility shift assays (EMSA) to detect STAT DNA binding activity in cells expressing the wild-type or the mutant STAT proteins

MCF-7 stable clones were seeded at $5-8 \times 10^6$ cells/100 mm and cultured in medium containing 0.5% FBS overnight. Cells were then untreated or treated with OM for 15 min. Nuclear extracts were prepared by the method of Dignam *et al* (1983) except that the buffer A was supplemented with 1 mM Na₃VO₄ and 1 μ g per ml each pepstatin and leupeptin. Nuclear extracts were quick frozen by liquid nitrogen and stored in aliquots.

Protein concentrations were determined using a modified Bradford assay using BSA as a standard (Pierce). EMSA and supershift assays were conducted as previously described (Li et al., 2001a,b,c) using a double-stranded oligonucleotide probe (c-FosSIE) containing the high-affinity STAT-binding site (m67) derived from the *c-fos* gene promoter (Wagner et al., 1990). A double-stranded oligonucleotide probe containing an SP1 binding site was used in the assay for the assessment of nonspecific bindings.

Transfection and reporter assays

Cells cultured in 24-well plates at a density of 0.12×10^6 cells per well were transiently transfected with a total of 200 ng of reporter DNA and 2 ng of pRL-SV40 (Renilla, Promega) per well mixed with the Effectene reagent. Cells were switched to medium containing 0.5% FBS overnight, stimulated with OM for indicated length of time, and harvested, 24 h after transfection. Luciferase activities in total cell lysates were measured using the Promega Dual Luciferase Assay System. Absolute firefly luciferase activity was normalized against renilla luciferase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed for each reporter construct. The STAT3 luciferase reporter constructs contain seven copies of specific STAT3 binding sites corresponding to the region -123/-85 of the *c-reactive protein* (CRP) promoter (Zhang et al., 1996). The p53 promoter luciferase reporter pGL3-p53 contains a 599 bp fragment of the human p53 promoter region and exon 1 (-426 to +172) (Li et al., 2001a,b,c).

Cell growth assay

Cell number count was conducted in monolayer culture in 24-well Costar culture plates. Cells were plated at an initial density of 7×10^3 cells/well in 0.5 ml medium supplemented with 2% FBS. OM was added 24 h after initial seeding. The culture media were replenished every 2 days. At the end of treatment, cells were trypsinized and then viable cell numbers were counted using a hemocytometer. Cell proliferation assay conducted in 96-well culture plates with an initial seeding of 2×10^3 cells/well was measured using CyQUANT Cell Proliferation Assay Kit (C-7026) obtained from Molecular Probes and a fluorescence microplate reader with the parameters of 480 nm excitation and 520 nm emission.

Western blot analysis

For detection of activated ERK, cells were cultured in medium containing 0.5% FBS for overnight prior to OM stimulation. For detection of cyclin D1, c/EBP δ , p53, and fibronectin expression, cells were cultured in medium containing 2% FBS with or without OM for various lengths of time. Cells in 60-mm culture dishes were lysed with 0.1 ml of cold lysis buffer (20 mM HEPES, pH 7.4, 30 mM *p*-nitrophenyl phosphate, 10 mM NaF, 10 mM MgCl₂, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na₃VO₄, 0.1 mM Na₂MnO₄, 10 mM Sodium B-glycerolphosphate, 10 mM Okadaic acid, 10 mM cypermethrin, 1 mM

phenylmethylsulfonyl fluoride, 5 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1.25 μ g/ml pepstatin). Approximately 50 μ g protein of total cell lysate per sample was separated on 10–15% SDS-PAGE, transferred to nitrocellulose membrane, followed by Western blot analysis. The signals detected using an enhanced chemiluminescence (ECL) detection system were quantitated with a BioRad Fluor-S MultiImager System. Densitometric analysis of autoradiographs in these studies included various exposure times to ensure linearity of signals.

RNA isolation and Northern blot analysis

Cells were lysed in Ultraspec RNA lysis solution (Biotecx Laboratory, Houston, TX, USA) and total cellular RNA was isolated according to the vendor's protocol. Approximately 15 μ g of each total RNA sample was used to analyse *c-myc* mRNA. The RNA blots were first hybridized to a 2 Kb human *c-myc* cDNA probe that was ³²P-labeled using the Primer-It II Random Primer Labeling kit (Stratagene) and then stripped and reprobed with a ³²P-labeled human GAPDH probe to ensure that equivalent amounts of RNA were being analysed. Hybridization signals were visualized by a BioRad Phosphor-Imager and were quantified by the Quantity One program.

Analysis of OM-induced morphological changes

MCF-7 and stable clones were cultured in the absence or presence of OM. At indicated times, cell morphology was examined under a phase contrast microscope equipped with a Penguin 600CL digital camera.

Migration assays

Cell motility was examined in a Boyden chamber assay using 8- μ m-pore polycarbonate membrane. The migrated cells were fixed and stained using the kit Hema 3 manual staining system obtained from Fisher Scientific. Cells were counted under an inverted microscope in 10 different $\times 100$ -power fields in triplicate wells.

Abbreviations:

ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; OM, oncostatin M; STAT, signal transducer and activator of transcription.

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References

- Badache A and Hynes N. (2001). *Cancer Res.*, **61**, 383–391.
- Bamber B, Reife R, Haugen H and Clegg C. (1998). *J. Mol. Med.*, **76**, 61–69.
- Bowman T, Broome M, Sinibaldi D, Wharton W, Pledger J, Sedivy J, Irby R, Yeatman T and Courtneidge, SJR. (2001). *Proc. Natl. Acad. Sci. USA*, **98**, 7319–7324.

- Bowman T, Garcia R, Turkson J and Jove R. (2000). *Oncogene*, **19**, 2474–2488.
- Brown TJ, Lionbin MN and Marquardt H. (1987). *J. Immunol.*, **139**, 2977–2983.
- Carroll J, Swarbrick A, Musgrove E and Sutherland R. (2002). *Cancer Res.*, **62**, 3126–3131.
- Catterall J, Carrere S, Koshy P, Degnan B, Shingleton W, Brinckerhoff C, Rutter J, Cawston T and Rowan A. (2001). *Arthritis Rheum.*, **44**, 2296–2310.
- Chang TL, Peng X and Fu X. (2000). *J. Biol. Chem.*, **275**, 10212–10217.
- Dignam JD, Lebovitz RM and Roeder RC. (1983). *Nucleic Acids Res.*, **11**, 1475–1489.
- Douglas AM, Goss GA, Sutherland RL, Hilton DJ, Berndt MC, Nicola NA and Begley CG. (1997). *Oncogene*, **14**, 661–669.
- Douglas AM, Grant SL, Goss GA, Clouston DR, Sutherland RL and Begley CG. (1998). *Int. J. Cancer*, **75**, 64–73.
- Duncan MR, Hasan A and Berman B. (1995). *J. Invest. Dermatol.*, **104**, 128–133.
- Grant SL, Hammacher A, Douglas AM, Goss GA, Mansfield R, Heath J and Begley C. (2002). *Oncogene*, **21**, 460–474.
- Grove RI, Eberhardt C, Abid S, Mazzucco CE, Liu J, Todaro GJ, Kiener PA and Shoyab M. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 823–827.
- Grove RI, Mazzucco CE, Allegretto N, Kiener PA, Spitalny G, Radka SF, Shoyab M, Antonaccio M and Warr GA. (1991). *J. Lipid Res.*, **32**, 1889–1897.
- Halfter H, Lotfi R, Westermann R, Young P, Ringelstein E and Stögbauer F. (1998). *Growth Factors*, **15**, 135–147.
- Halfter H, Stögbauer F, Friedrich M, Serve S, Serve H and Ringelstein E. (2000). *J. Neurochem.*, **75**, 973–981.
- Heinrich PC, Behrmann I, Muller-Newen G, Schaper F and Graeve L. (1998). *Biochem. J.*, **334**, 297–314.
- Hirano T, Nakajima K and Hibi M. (1997). *Cytokine Growth Factor Rev.*, **8**, 241–252.
- Horn D, Fitzpatrick WC, Gompper PT, Ochs V, Bolton-Hanson M, Zarling JM, Malik N, Todaro GJ and Linsley PS. (1990). *Growth Factors*, **2**, 157–165.
- Hutt J, O'Rourke J and DeWille J. (2000). *J. Biol. Chem.*, **275**, 29123–29131.
- Kelly K, Cochran BH, Stiles CD and Leder P. (1983). *Cell*, **35**, 603–610.
- Kerr C, Langdon C, Graham F, Gauldie L, Hara T and Richard CD. (1999). *J. Interferon Cytokine Res.*, **19**, 1195–1205.
- Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, Mizuno K, Hibi M and Hirano T. (1999). *J. Exp. Med.*, **189**, 63–73.
- Kortylewski M, Heinrich P, Mackiewicz A, Schniertshauer U, Klingmüller U, Nakajima K, Hirano T, Horn F and Behrmann I. (1999). *Oncogene*, **18**, 3742–3753.
- Leaman D, Leung S, Li X and Stark G. (1996). *FASEB J.*, **10**, 1578–1588.
- Li C, Ahlborn TE, Kraemer FB and Liu J. (2001a). *Breast Cancer Res. Treat.*, **66**, 111–121.
- Li C, Ahlborn TE, Tokita K, Boxer L, Noda A and Liu J. (2001b). *Oncogene*, **20**, 8193–8202.
- Li W, Dehnade F and Zafarullah M. (2001). *J. Immunol.*, **166**, 3491–3498.
- Li W, Liang X, Kellendonk C, Poli V and Taub R. (2002). *J. Biol. Chem.*, **1**, 1–10.
- Liu J, Clegg JC and Shoyab M. (1992). *Cell Growth Differ.*, **3**, 307–313.
- Lin J, Jin X, Rothman K, Liu H, Tang H and Burke W. (2002). *Cancer Res.*, **62**, 376–380.
- Liu J, Li C, Ahlborn TE, Spence MJ, Meng L and Boxer LM. (1999). *Cell Growth Differ.*, **5**, 15–18.
- Liu J, Spence MJ, Wallace PM, Forcier K, Hellstrom I and Vestal RE (1997). *Cell Growth Differ.*, **8**, 667–676.
- Mahboubi K and Pober J. (2002). *J. Biol. Chem.*, **277**, 8012–8021.
- Makogonenko E, Tsurupa G, Ingham K and Medved L. (2002). *Biochemistry*, **41**, 7907–7913.
- Marsters P, Morgen K, Morley S, Gent D, Hejazi A, Backx M, Thorpe E and Kalsheker N. (2002). *Biochem. J.*, **1**, 1–10.
- Minami M, Inoue M, Wei S, Takeda K, Matsumoto M, Kishimoto T and Akira S. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 3963–3966.
- Noda A, Toma-Aiba Y and Fujiwaba Y. (2000). *Oncogene*, **19**, 21–31.
- Rayanade RJ, Ndubuisi MI, Etlinger JD and Sehgal PB. (1998). *J. Immunol.*, **161**, 325–334.
- Rayanade RJ, Patel K, Ndubuisi M, Sharma S, Omura S, Etlinger JD, Pine R and Sehgal PB. (1997). *J. Biol. Chem.*, **272**, 4659–4662.
- Sauter E, Yeo U, SteMM A, Zhu W, Litwin S, Tichansky D, Pistritto G, Nesbit M, PiNkel D, Herlyn M and Bastian B. (2002). *Cancer Res.*, **62**, 3200–3206.
- Schaefer L, Wang S and Schaefer T. (2000). *Cytokine*, **12**, 1647–1655.
- Sherr C and Roberts J. (1999). *Genes Dev.*, **13**, 1501–1512.
- Sinibaldi D, Wharton W, Turkson J, Bowman T, Pledger W and Jove R. (2000). *Oncogene*, **19**, 5419–5427.
- Spence MJ, Vestal RE and Liu J. (1997). *Cancer Res.*, **57**, 2223–2228.
- Strand K, Murray J, Aziz S, Ishida A, Rahman S, Patel Y, Cardona C, Hammond W, Savidge G and Wijelath E. (2000). *J. Cell. Biochem.*, **79**, 239–248.
- Wagner B, Hayes T, Hoban C and Cochran B. (1990). *EMBO J.*, **13**, 4477–4484.
- Wahl A and Wallace PM. (2001). *Ann. Rheum. Dis.*, **60**, iii75–iii80.
- Yamada T, Tobita K, Osada S, Nishihara T and Imagawa M. (1997). *J. Biochem.*, **121**, 731–738.
- Zarling JM, Shoyab M, Marquardt H, Hanson MB, Lionbin MN and Todaro GJ. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 9739–9743.
- Zhang XG, Gu JJ, Lu ZY, Yasukawa K, Yancopoulos GD, Turner K, Shoyab M, Taga T, Kishimoto T, Bataille R and Klein B. (1994). *J. Exp. Med.*, **179**, 1343–1347.
- Zhang D, Sun M and Samols D. (1996). *J. Biol. Chem.*, **271**, 9503–9509.



Molecular characterization of oncostatin M-induced growth arrest of MCF-7 cells expressing a temperature-sensitive mutant of p53

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Key words: cell cycle regulation, microarray analysis, oncostatin M, p21, p53

Summary

Our previous studies have shown that treatment of MCF-7 breast cancer cells with cytokine oncostatin M (OM) results in a growth arrest and a concurrent decrease in p53 expression. It remains to be determined whether these two important events are directly connected, as changes in p53 protein levels can lead to variable biological outcomes. In this study we have generated stable cell lines (MCF7-ptsp53) that express p53Val¹³⁵ a p53 temperature-sensitive mutant. We demonstrate that overexpression of the wildtype (wt) p53 at permissive temperature in MCF7-ptsp53 cells leads to growth arrest at the G₂-M phase of the cell cycle. Inhibition of endogenous p53 function with the expression of mutant p53 protein at non-permissive temperature did not affect the OM-induced G₁ cell cycle arrest. Microarray studies were further carried out to identify p53- and OM-regulated genes that mediate the G₂/M or G₁ cell cycle arrest. We show that the expression of p21 was upregulated and expressions of cdc2, cyclin B2 and protein regulator of cytokinesis 1 (PRC1) were suppressed by overexpression of the wt p53 in MCF7-ptsp53 cells at the permissive temperature. In contrast, OM treatment caused coordinate changes of mRNA expression of several cell cycle components including c/EBP δ , cdc20, and thymidine kinase 1 (TK1) that mainly affect G₁-S phase transition. All together, our results suggest that the downregulation of p53 transcription may be involved in some other cellular changes induced by OM but it is not directly connected to the antiproliferative activity of OM *per se*.

Abbreviations: CDC2: cell division cycle 2; FBS: fetal bovine serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TS: temperature sensitive; OM: oncostatin M; PI: propidium iodide; PRC1: protein regulator of cytokinesis 1; TK1: thymidine kinase 1

Introduction

Oncostatin M (OM), a 28 kDa glycoprotein, is a cytokine produced by activated T lymphocytes and macrophages [1]. Previous studies showed that OM inhibits the growth of several breast cancer cell lines. OM-treated breast cancer cells show a reduced growth rate that is accompanied by an increased proportion of cells in G₀/G₁ phase and a concomitant decrease in the number of cells in S phase. In addition, a variety of morphological changes associated with the differentiated phenotype appeared after OM treatment [2–4]. However, OM treatment does not lead to apparent apoptosis. Since the p53 tumor suppressor protein plays important roles in cellular proliferation, differentiation

and apoptosis [5], we examined the effects of OM on p53 expression in breast cancer cells. Surprisingly, we found that p53 expression was down regulated by OM in MCF-7 cells that express the wt functional p53 [6]. Treatment of MCF-7 cells with OM for 5 days reduced p53 protein expression by more than 50% as compared to the control and the viable cell numbers were concurrently decreased by about 70%. In addition to OM, we found that the p53 expression in MCF-7 cells was also suppressed by PMA, a differentiation-inducing agent for these cells. Further investigations demonstrate that the down regulation of p53 protein as well as mRNA expressions by OM occurs at the transcriptional level and is mediated through a specific OM-responsive *cis*-acting element (PE21) of the p53 promoter [7].

Pdf Output

Based upon these observations, we initially hypothesized that p53 plays a positive role in apoptosis, but it could have a negative role in the differentiation process in breast tumor cells. Decreasing p53 expression might be a factor that contributes to the cell commitment to undergo growth arrest/differentiation and to prevent apoptosis in response to OM exerted stress. In order to investigate the relationship between p53 expression and the growth inhibition of breast cancer cells, and to determine whether p53 plays a negative role in the OM-induced growth arrest of breast cancer cells, in this study, we utilized the temperature-sensitive (ts) p53 mutant p53Val¹³⁵ to establish a cell system where the transactivating function of p53 can be turned on or turned off by culturing cells at either permissive temperature 32°C or at the non-permissive temperature 37°C [8–10]. It has been well documented that at 37°C p53Val¹³⁵ transfectants express exogenous p53 in a mutant conformation that acts as a dominant negative mutant and inhibits the function of the endogenous p53 [11, 12]. In contrast, at permissive temperature 32°C, the p53Val¹³⁵ mutant resumes normal conformation and behaves as the wt p53. Previous reports in literature showed that ectopic expression of the ts p53 mutant had different effects on cell cycle in different cell lines. In many cases, cells expressing the p53Val¹³⁵ were arrested primarily in the G₀/G₁ phase of the cell cycle, suggesting that p53 plays a specific role at G₁ [11–15]. It has been shown that transfection of p53Val¹³⁵ into Hs578T human breast cancer cells resulted in the G₁ cell cycle arrest [13]. However, a few reports had showed that a second cell cycle block at G₂/M occurred in human fibroblasts by overexpression of the wt p53 [16, 17]. This suggests that p53 may have an important role in regulating the G₂–M transition. In addition, p53Val¹³⁵ overexpression at 32°C induces apoptosis in Jurkat [18] and U-937 cells [19]. Presently, it is not totally clear what factors are involved in the determination of cell fate in response to p53 overexpression.

In this study, we show that expression of p53Val¹³⁵ at permissive temperature in MCF-7 cells led to a growth arrest mainly at the G₂/M phase. Exposure of MCF7-ptsp53 cells to OM at the non-permissive temperature that inhibited the endogenous wt p53 transactivation function did not affect the OM-induced G₁ arrest nor induced an onset of apoptosis. These results suggest that the down regulation of p53 transcription may be involved in some other cellular changes induced by OM but it is not directly connected to the antiproliferative activity of OM *per se*.

Materials and methods

Cells and reagents

Human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Purified human recombinant OM was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). The plasmid ptsp53-Val¹³⁵ (pLTRp53cGVal135) was kindly provided by Dr Moshe Oren at Weizmann Institute of Science, Rehovot, Israel. The plasmid p53Luc that contains five binding sites for p53 was purchased from Stratagene. Anti-murine p53 antibody (Pab246), anti-p21, and anti-cyclin B2 were purchased from Santa Cruz Biotechnology, Inc.

Generation of stable clones (MCF7-ptsp53) expressing p53Val¹³⁵

To generate stable MCF-7 clones that constitutively express p53Val¹³⁵ mutant, plasmid pLTRp53cGVal¹³⁵ and an empty vector (pcDNA3.1) containing a neomycin resistant gene were co-introduced into MCF-7 cells cultured at 37°C using the transfection reagent Effectene (Qiagen, Valencia, CA). Cells were selected in medium containing 300 µg/ml G418. Several clones were picked, expanded in the presence of G418. The expression of p53Val¹³⁵ in selected clones was examined by immunostaining and western blot with anti-p53 antibody (Pab246) that only recognizes the mutant murine p53 but not the endogenous human p53.

Western blot analysis

Cells in 60 mm culture dishes were lysed with 0.1 ml of cold lysis buffer [6] supplemented with 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals). Approximately 30 µg protein of total cell lysate per sample was separated on 10–15% SDS PAGE, transferred to nitrocellulose membrane, followed by western blot analysis. After probing the target proteins, membranes were stripped and reblotted with anti-β-actin antibody for detection of the difference in sample loading. The signals detected using an enhanced chemiluminescence (ECL) detection system were quantitated with a BioRad Fluor-S MultiImager System. Densitometric analysis of auto-

radiographs in these studies included various exposure times to ensure linearity of signals.

Reporter assays to determine the p53 transactivating activity

MCF-7 cells and stable clones were plated at a density of 8×10^4 cells/well in 24-well plates and incubated for 24 h at 37 or 32°C in medium containing 10% FBS before transfection. Cells were transiently transfected with a total of 200 ng of reporter DNA (p53Luc) and 2 ng of pRL-SV40 (Renilla, Promega) per well mixed with the Effectene reagent. Transfected cells were incubated at 37 or 32°C for 40 h prior to cell lysis. Luciferase activities were measured using the Promega Dual Luciferase Assay System.

Cell growth assay

Viable cell number count was conducted in monolayer culture in 24-well costar culture plates. Cell proliferation assay to determine total DNA content was conducted in 96-well culture plates using CyQUANT Cell Proliferation Assay Kit (C-7026) obtained from Molecular Probes and a fluorescence microplate reader with the parameters of 480 nm excitation and 520 nm emission.

Flow cytometric analysis

Distribution of DNA content in MCF-7 cells and stable clones was determined by flow cytometry. Cells were fixed in 70% ethanol for 2 h at 4°C. After washing with PBS, cells were resuspended in PBS containing propidium iodide (PI) (50 µg/ml) and DNase-free RNase A (50 µg/ml) and were incubated at room temperature for 20 min in dark. Forward and orthogonal scatter lights as well as red fluorescence was analyzed by fluorescence-activated cell sorting (FACS). A dual parameter dot plot of the integrated area versus the width of the fluorescence pulse was displayed to exclude cell aggregates from cell counting and analysis. The histogram of DNA distribution was modeled as a sum of G₁, G₂-M and S phase by using ModFitLT software.

Apoptosis assay

The Vybrant™ Apoptosis Assay Kit (Molecular Probes) was used to detect apoptotic cells in MCF-7 and MCF7-ptsp53 cells during temperature shift and during OM treatment. Briefly, MCF-7 and MCF7-ptsp53 were seeded 4000 cells per well in 96 black

wall plates in RPMI-1640 medium containing 10% FBS. Duplicated plates were set up, one set was cultured at 37°C and the other set was cultured at 32°C. At indicated times, culture medium was removed and cells were washed once with PBS. Then YO-PRO-1 dye and PI (1 µl/ml) in PBS were added to the cells. The detection of apoptotic cells (green) and dead cells (red and green) were performed with microscope and with a fluorescence microplate reader to measure the relative fluorescence intensities. For YO-PRO-1 dye, the Ex/Em is 491 nm/509 nm; for PI, the Ex/Em is 535 nm/617 nm. To examine the appearance of apoptotic cells in MCF-7 and MCF7-ptsp53 clones after exposure to OM, cells were cultured in the presence or absence of OM at both 37 and 32°C for several days. Apoptotic assays were performed at different intervals.

Analysis of cell morphological changes

MCF-7, MCF7-neo, or MCF7-ptsp53 were treated with OM or treated by temperature switch. At the indicated times, cell morphology was examined under a phase contrast microscope equipped with a Penguin 600CL digital camera.

Microarray analysis

The human cDNA array containing some 40–45K genes was obtained from the Microarray Core Facility at Stanford University School of Medicine. Detailed protocols for probe labeling and hybridization are available at the Stanford web sites (<http://cmgm.stanford.edu/pbrown/>, <http://genome-www.stanford.edu/molecularportraits/>). Briefly, untransfected MCF-7 cells and MCF7-ptsp53 cells in RPMI-1640 with 10% FBS were cultured at 37 or 32°C for 4 days. Total RNA was then isolated by RNAeasy kit (Qiagen). Sixty micrograms of total RNA per sample was used to generate the first-strand cDNA probe in the presence of Cy3-dCTP (for cells at 37°C) or Cy5-dCTP (for cells at 32°C) in the reaction of reverse transcription. Labeled cDNA probes were purified using Micron YM-30 column (Millipore), denatured, and hybridized to an arrayed slide overnight at 65°C. Slide was washed in 2× SSC/0.1% SDS for 2 min, 2× SSC 1 min, 1× SSC 1 min, and in 0.1× SSC for 1 min before air-dry. The fluorescent images were captured using a GenePix 4000 scanner (Axon Instruments, Foster city, CA). To analyze the array data, the CLUSTER program was applied to obtain an average-linkage hierarchical clustering

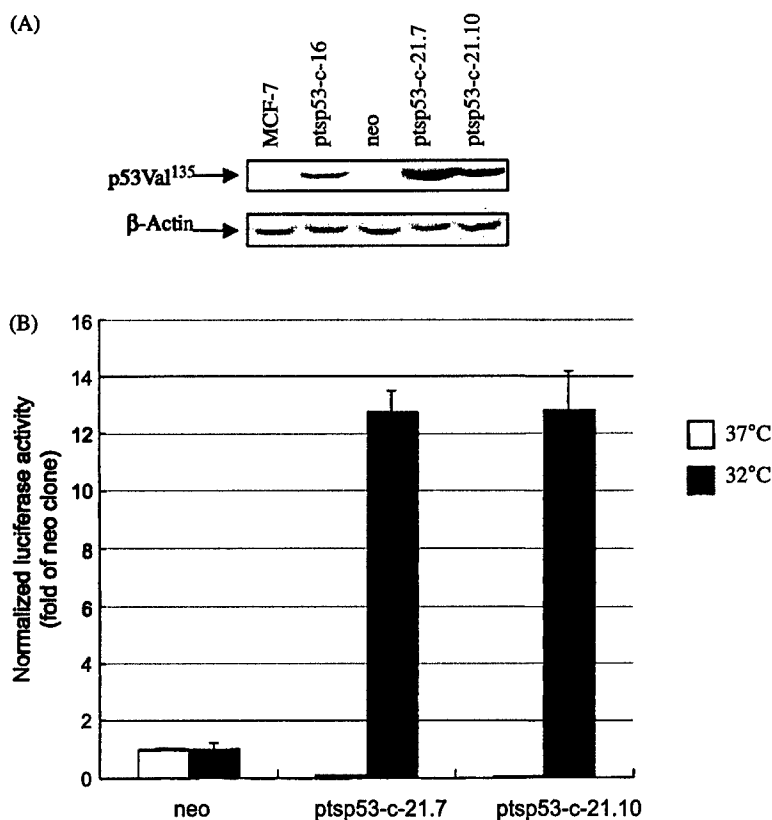


Figure 1. Characterization of MCF7-ptsp53 stable clones. (A) Western blot analysis of the murine p53Val¹³⁵ protein expression. Cell lysates were harvested from MCF-7, neo clone, and ptsp53 clones. Soluble proteins (50 µg/lane) were applied to SDS-PAGE. Detection of p53Val¹³⁵ mutant protein was conducted by immunoblotting with anti-murine p53 mAb (pAb246). (B) Luciferase reporter assays to determine p53 transactivating activity in stable clones. Cells were cultured at either 37 or 32°C overnight and were thereafter transfected with plasmids p53-Luc and pRL-SV40. Total cell lysates were harvested 40 h after transfection. The firefly and renilla luciferase activities were measured. The normalized firefly luciferase activity is expressed as the fold of luciferase activity in control cells (neo). The data presented are representative of three to five separate transfections in which triplicate wells were used in each condition.

and the results were displayed by using the program TREEVIEW (software available at <http://genome-www4.stanford.edu/MicroArray/SMD/restech.html>). The fluorescent intensities of cy-5 and cy-3 for each target spot were automatically adjusted by the analyzing program in a way that the house keeping genes for each slide became equal. The genes whose expression varied by at least 2-fold from the median red/green rating were subsequently selected and grouped according to their primary functions defined by GeneCards (<http://bioinformatics.weizmann.ac.il/cards/>).

RT-PCR analysis

Total RNAs were isolated from MCF-7 and MCF7-ptsp53 cells cultured either at 37 or 32°C. First strand

cDNA was synthesized by using the Superscript II RNase H-Reverse Transcriptase Kit (Invitrogen) and 1 µg of total RNA in a volume of 20 µl per reaction. PCR reactions using 1 µl of RT reaction product with a total of 30 cycles were performed for cyclin B2, yielding a product of 351 bp fragment. PCR of 26 cycles was conducted for GAPDH. The PCR conditions were 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s with a final extension at 72°C for 5 min.

The sequences of primers are as follows:

- Cyclin B2 forward primer, 5'-AAAGTTGGCTCC AAAGGGTCCTT-3'.
- Cyclin B2 reverse primer, 5'-GAAACTGGCTG AACCTGTAAAAAT-3'.

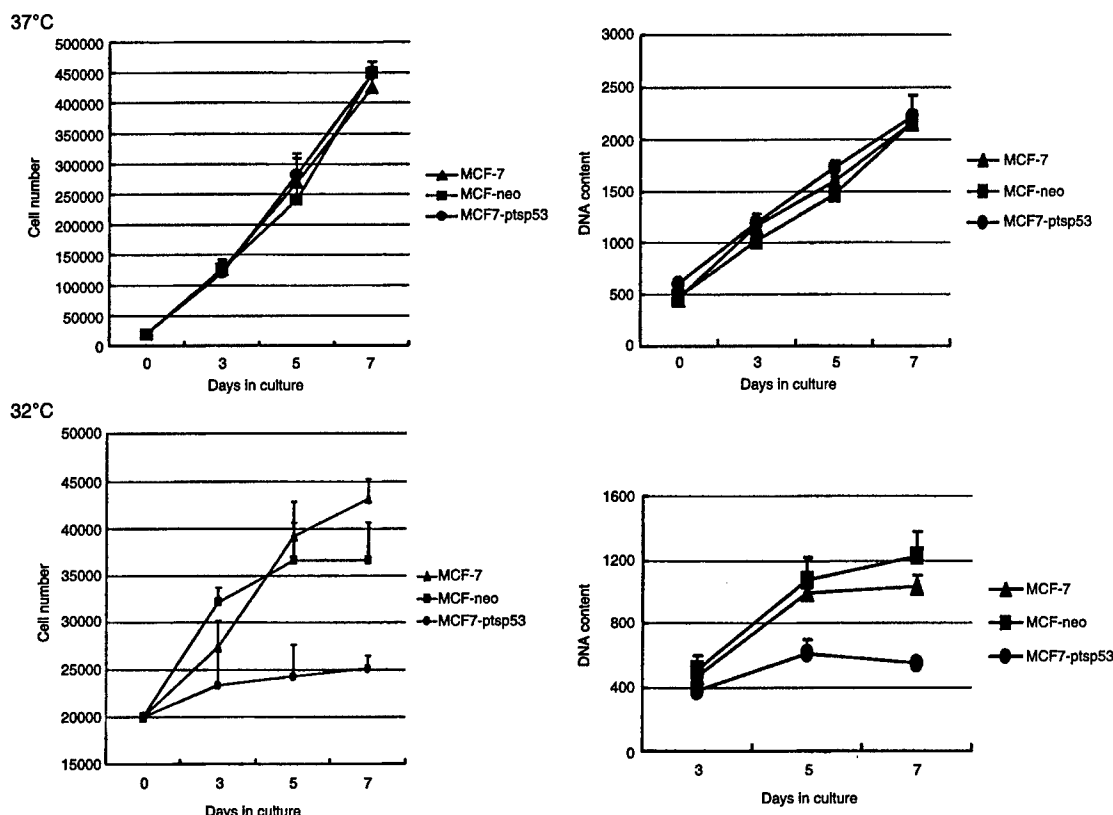


Figure 2. Examination of the effect of p53Val¹³⁵ on the growth rate of MCF-7 cells at non-permissive and the permissive temperature. Left panel: Cell number account – Cells of MCF-7 and stable clones were cultured at a density of 2×10^4 cells/well in 24-well culture plates at either 37 or 32°C for different days. At the indicated time, cells were trypsinized and viable cells (trypan blue excluding cells) were counted. Values are mean of triplicate wells. Right panel: DNA content – Cells were cultured in 96-well plates at a density of 2000 cells/well in 0.1 ml RPMI containing 10% FBS for different intervals. At the indicated time, the medium was removed and cells were washed with PBS. Two hundred microliters of Cyquant GR dye mixed with cell lysis buffer were added to each well. The fluorescent signals were then measured using a fluorescence microplate reader. Values are mean of triplicate wells. The graph shown is representative of at least three separate experiments with consistent results.

- GAPDH forward primer, 5'-CCATCACTGCCAC CCAGAAGAC-3'.
- GAPDH reverse primer, 5'-GGCAGGTTTTTCTA GACGGCAG-3'.

Results

Manipulation of p53 transactivating function by temperature switch

For establishing a system to study the effects of p53 on cell growth, we developed stable MCF-7 clones that express a ts p53 mutant (p53Val¹³⁵) by cotransfection of the plasmid pLTRp53cGVal¹³⁵ and pcDNA3.1. MCF-7 clones (neo) transfected with the selection vector (pcDNA3.1) were also generated and were used in

this study as negative controls to access possible side effects associated with antibiotic selection. Western blot using anti-murine p53 antibody was conducted to examine the expression of the p53 mutant in selected clones. A total of 12 independent positive clones were selected. Figure 1(A) shows high levels of p53Val¹³⁵ expression in three of the representative clones. As expected, the p53 mutant was not detected in untransfected or mock-transfected MCF-7 cells.

To determine the effect of exogenous expressed p53Val¹³⁵ on the function of endogenous wt p53, we compared the p53 transactivating activity in MCF7-neo and in MCF7-ptsp53 clones cultured either at 37 or 32°C by transient transfection of a specific p53 luciferase reporter (p53Luc). Temperature shift from 37 to 32°C had no effect on p53 activated expression of luciferase gene in neo clone, but it markedly

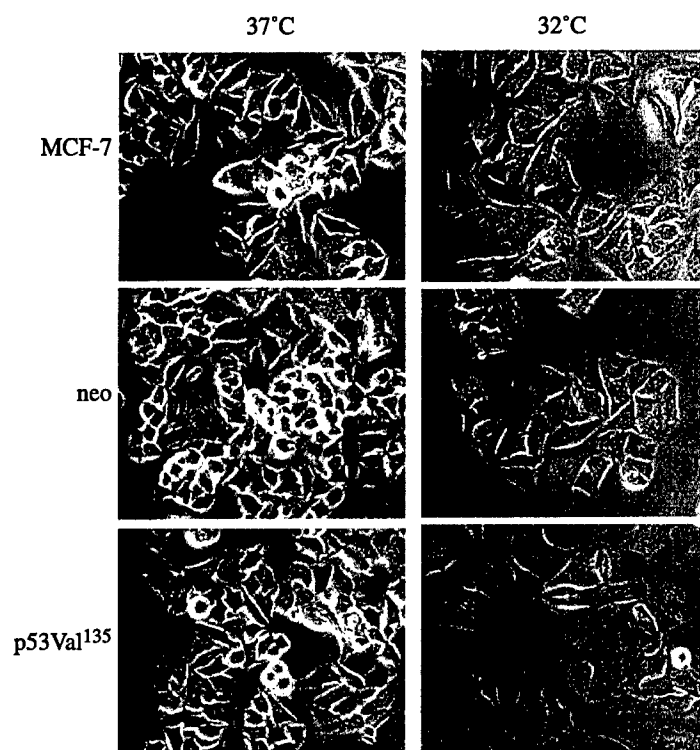


Figure 3. Overexpression of p53 induces morphological changes in MCF-7 cells. MCF-7, the neo clone, and the ptsp53 clone were cultured at 37 or 32°C for 3 days and photographs were taken using the Penguin 600CL digital camera at a magnification of 200 \times .

affected the transactivating activity of p53 in ptsp53 clones (Figure 1(B)). At 37°C the luciferase activities in ptsp53 clones were decreased to levels of less than 5% of the neo clone whereas at 32°C the luciferase activities were increased approximately 13-fold of the neo clone. These data clearly demonstrate that at the non-permissive temperature ptsp53 acted as a dominant negative mutant that completely abolished the transactivational function of the endogenous wt p53 in MCF-7 cells. By contrast, at 32°C the permissive temperature, ptsp53 resumed its native conformation and activated gene transcription through the binding to the canonical sites upstream of the luciferase gene, resembling the wt p53.

Overexpression of wt p53 leads to growth arrest without induction of apoptosis or senescence

We first characterized the effects of p53 overexpression on cell growth. MCF-7, MCF7-neo, and MCF7-ptsp53 cells were seeded in culture plates at 37°C overnight. On next day one set of plates was switched

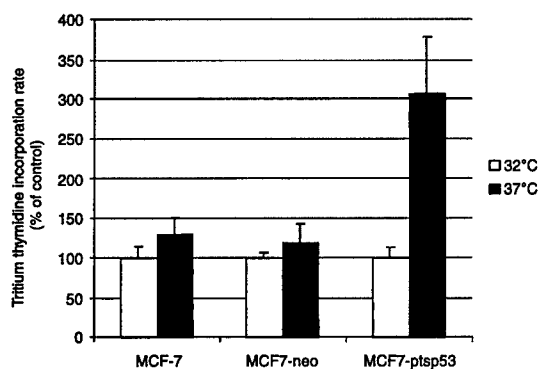


Figure 4. Reversal of p53-induced growth arrest by temperature switch. MCF-7, MCF7-neo, MCF7-ptsp53 cells were cultured in 24-well plates at 32°C for 2 days. Then one set of plates was incubated at 37°C and the other set was remained at the 32°C. Cells were then pulsed with [3 H]thymidine for 16 h. The amount of radioactivity incorporated into cells was determined by TCA precipitation assay [29]. Data are expressed as the percentage of radioactivity incorporated in cells at 37°C relative to cells at 32°C for each cell line.

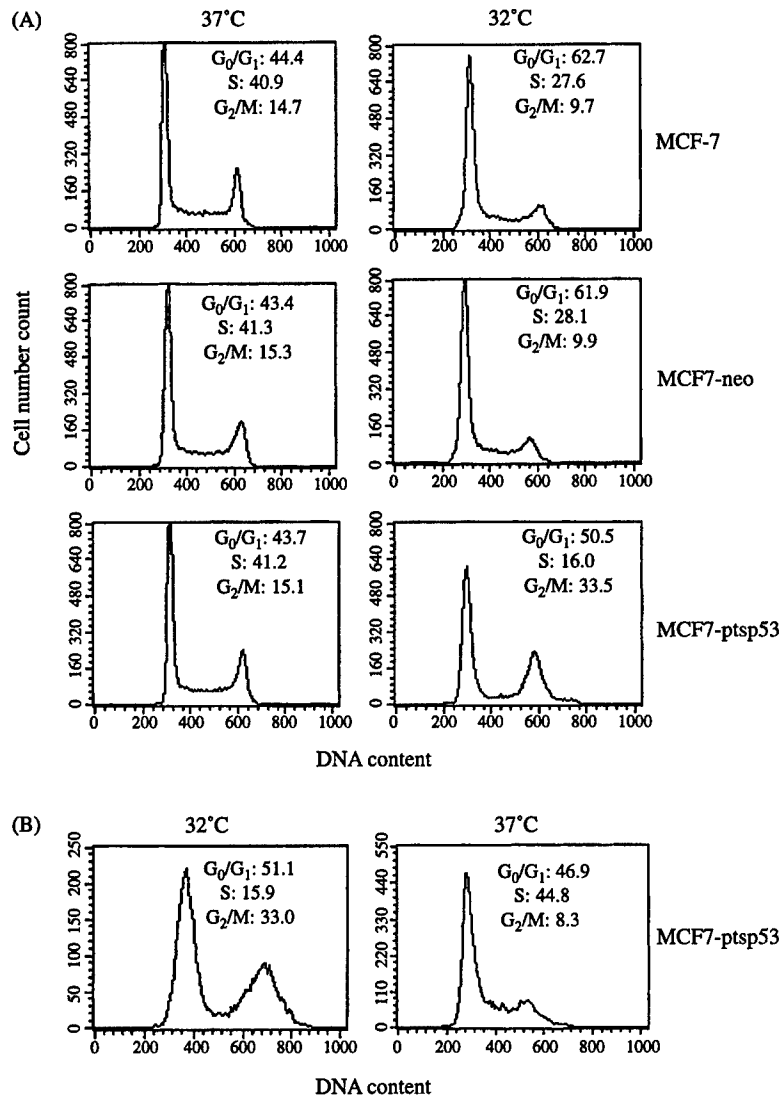


Figure 5. Cell cycle distribution by FACS analyses. In (A), MCF-7, MCF7-neo, MCF7-ptsp53 cells seeded at equal densities were cultured for 2 days at 37°C and then were switched to 32°C. Cells were harvested for flow cytometry on days 0 and 2 after the temperature shift down. In (B), MCF7-ptsp53 cells cultured at 32°C were switched to 37°C and cells were harvested for flow cytometry on days 0 and 2 after the temperature shift up. Values show percentage of viable cells.

to 32°C while another set was continuously cultured at 37°C. Cells were harvested at indicated intervals and the cell proliferation rate was determined by direct accounting the viable cell number after detachment with trypsin (left panel) and by measuring the DNA content (right panel). The upper panels of Figure 2 show that at 37°C, the growth rates of MCF-7 and the stable clones were identical, regardless of the p53 status. In con-

trast, at 32°C the proliferation rate of ptsp53 clone was markedly reduced while the mock clone and MCF-7 cells continued to grow albeit at a slightly slower rate as compared to 37°C (Figure 2, lower panels). Microscopic examination shows that at 37°C the morphologies of parental MCF-7, mock-transfected, and ptsp53-transfected cells are indistinguishable, whereas at 32°C the size of MCF7-ptsp53 cells was signifi-

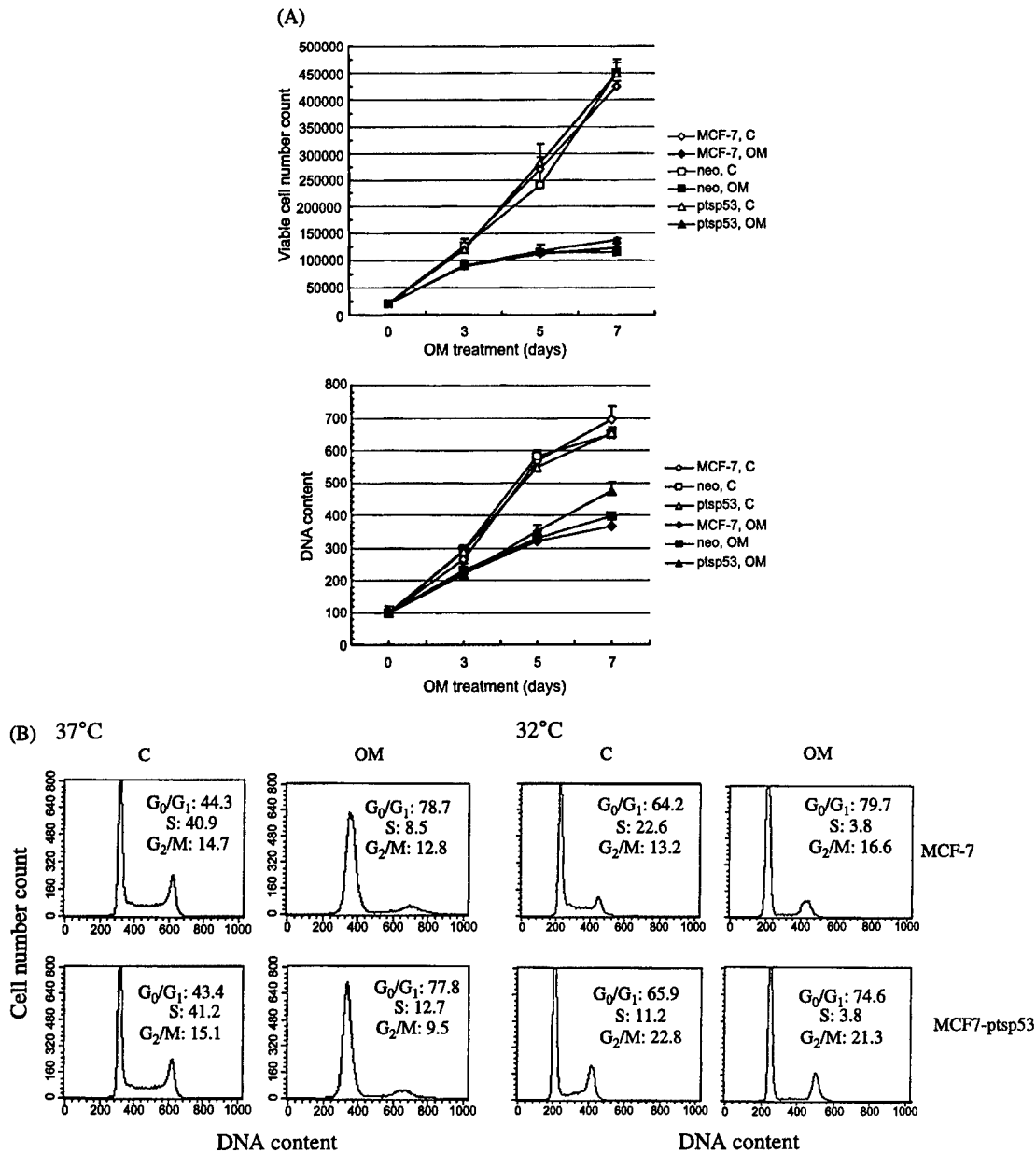


Figure 6. OM induces growth G₁ arrest in MCF-7 and MCF7-ptsp53 cells. (A) Cell proliferation assays: Cells of MCF-7 and stable clones were cultured at 37°C in medium containing 2% FBS with or without 50 ng/ml of OM for different days. At the indicated time, cell proliferation rates were determined by counting of the viable cell numbers (left panel) and by measuring the DNA content (right panel) as described in Figure 2. (B) Flow cytometry analysis: Cells were cultured in the presence or absence of OM (50 ng/ml) at either 37 or 32°C. Cell cycle distribution was determined by FACS analyses. The data shown are representative of three separate experiments. (C) Examination of OM-induced morphological changes in MCF-7 and MCF7-ptsp53 cells cultured at 32°C. The OM-induced morphological changes were also observed in MCF7-ptsp53 cells when cultured at 37°C.

cantly larger and flatter than control cells (Figure 3). Characteristic features of apoptosis such as reduced cell size, condensed nuclei, and fragmented nuclei

were not observed in any of the ptsp53 positive clones even after several days of culturing at 32°C. The lack of apoptotic cells was also proved by the negative

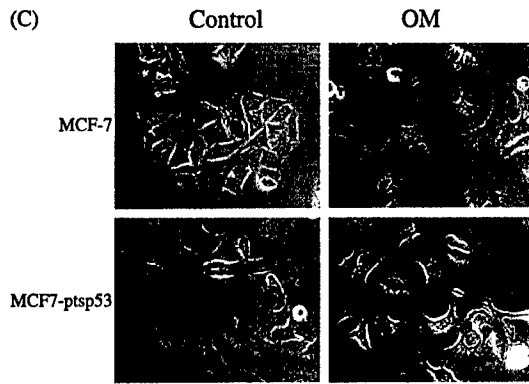


Figure 6. (continued)

staining of ptsp53 cells with YO-PRO-1 dye that turns apoptotic cells green under fluorescence.

The morphologically altered phenotype and the growth inhibition could result from senescence induced by overexpression of wt p53 [20, 21]. We performed the SA β -galactosidase staining in these cells to detect the appearance of senescence and did not observe a notable difference in the numbers of positive-stained cells (<1%) no matter at 37 or 32°C. The absence of p53-induced senescence in ptsp53 cells was further confirmed by an assay of ^3H -thymidine incorporation that showed a stimulated DNA synthesis of ptsp53 cells by temperature shift-up from 32 to 37°C (Figure 4). Therefore, we conclude that overexpression of wt p53 results in a reversible growth arrest.

G₂-specific cell cycle arrest

Increased expression of wt p53 has been shown to arrest cells at the G₀/G₁ and/or G₂/M. Expression of p53Val¹³⁵ in Hs578T human breast cancer cells was shown to cause a complete G₁ cell cycle arrest [13]. To examine MCF-7 cells for changes in cell cycle progression associated with wt p53 expression, MCF-7, MCF7-neo, MCF7-ptsp53 cells seeded at equal densities were cultured for 2 days at 37°C and then were switched to 32°C and harvested for flow cytometry on days 0 and 2 after the temperature change. Figure 5(A) shows that the temperature switch had only a small effect on the cell cycle progression of MCF-7 (upper panel) and the neo clone (middle panel) by a slight increase in the number of cells in the G₁ phase. This likely reflected an overall slower growth rate of these cells at the lower temperature. However, the temperature shift specifically changed the cell cycle

distribution of MCF7-ptsp53 cells (lower panel) by a significant increase in cells in the G₂ phase (day 0: 15.1% v.s. day 2: 33.5%) and a concomitant considerable decrease in the percentage of cells in the S phase (day 0: 41.2% v.s. day 2: 16.0%), while the percentage of cells in the G₁ phase was not appreciably increased (day 0: 43.7% v.s. day 2: 50.4%). Conversely, switching MCF7-ptsp53 cells from 32 to 37°C decreased G₂/M phase from 33 to 8.3% with a concurrent increase of S phase from 15.9 to 45% without a notable change in the G₀/G₁ phase (Figure 5(B)). These data together strongly suggest that overexpression of the wt p53 in MCF7-ptsp53 cells mainly affects the G₂/M phase of the cell cycle.

Blockade of endogenous p53 function neither abrogated the OM-induced G₁ growth arrest nor induced apoptosis

Having established ptsp53 cell lines in which the endogenous p53 transactivating function can be inhibited, we compared the effects of OM on the growth rates and cell morphologies of MCF-7, MCF7-neo, and MCF7-ptsp53. The results in Figure 6(A) shows that OM exerted a strong antiproliferative activity on MCF-7 and stable clones no matter the cells express the wt p53 or the p53 dominant negative mutant (ptsp53). The results of cell cycle analysis indicated that OM treatment significantly decreased the percentage of cells in S phase and increased the numbers of cells in G₁ phase in all cell lines cultured at 37°C. The G₂/M phase was not significantly affected by OM. Although we could not clearly demonstrate the growth inhibitory activity of OM in MCF7-ptsp53 cells at 32°C due to the p53-induced growth arrest, the flow cytometric analysis showed a similar pattern with slightly increased G₁ phases and decreased S phases in MCF-7 and MCF7-ptsp53 cells (Figure 6(C)).

By utilizing the YO-PRO-1 dyes, we looked for the appearance of apoptotic cells by microscopic examination and by quantitative spectrometric measurement. In these assays, we used okadaic acid as a positive control. While more than 50% of cells underwent apoptosis after 4 h exposure to okadaic acid at a concentration of 400 nM, no significant increase of apoptotic cells or necrotic cells was detected in OM-treated MCF-7, neo, and ptsp53 clones during the entire experimental period (8 h to 5 days) no matter cells were cultured at 37 or 32°C. This is consistent with the FACS analysis of lacking the sub-G₀ apop-

Table 1. Genes regulated by overexpressed p53

No.	Accession no.	Gene name	MCF7-ptsp53 at 32°C versus MCF7-ptsp53 at 37°C (ratio)	MCF7-ptsp53 at 32°C versus MCF-7 at 37°C (ratio)	MCF-7 at 32°C versus MCF-7 at 37°C (ratio)
Cell growth					
1	N23941	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	11.9	9.6	1.4
2	AA450062	Prostate differentiation factor	3.7	7.2	1.0
3	AI955990	PRG4 proteoglycan 4	0.5	0.2	1.2
4	AA598974	Cell division cycle 2 (CDC2)	0.5	0.2	0.6
5	AA449336	Protein regulator of cytokinesis 1 (PRC1)	0.3	0.2	1.4
6	AI932735	Cyclin B2	0.3	0.2	0.6
7	AA911194	Melanoma differentiation associated protein-5 (MDA5)	0.2	0.3	1.0
8	AI357590	2'-5'-oligoadenylate synthetase 3 (OAS3)	0.1	0.4	0.8
9	AA481164	2'-5'-oligoadenylate synthetase 2 (OAS2)	0.1	0.3	1.1
Chaperone protein					
10	AW004895	Heat shock 60 kDa protein 1 (HSK60-1)	0.4	0.2	1.1
Metabolism					
11	AA455800	Gamma-glutamyl hydrolase (GGH)	0.3	0.2	0.8
Signaling pathway					
12	N59336	Solute carrier family 7, member 11 (SLC7A11)	0.3	0.2	0.8
13	H81023	Serine/threonine kinase 12 (STK12)	0.2	0.4	0.9
14	AA076085	Signal transducer and activator of transcription 1 (STAT1)	0.2	0.3	1.1

otic population in OM-treated MCF-7 and the stable clones.

Figure 6(C) shows OM-induced morphological changes in MCF-7 and MCF7-ptsp53 cells. The typical changes include disruption of cell to cell junctions, enlarged cytoplasm, and appearance of cell extensions and membrane protrusions. Although ptsp53 cells are larger and flatter as compared to MCF-7 cells in the absence of OM, the OM-induced membrane protrusion and cell extension were clearly seen in both cell lines. All together, these results suggest that in MCF-7 cells, elimination of the transcriptionally active p53 did not directly affect OM activities in the regulation of cell growth and morphology.

Identification of p53-regulated genes mediating G₂/M cell cycle arrest by microarray analysis

We were interested in identifying p53-regulated genes that participate in the control of cell cycle progression. To accomplish this, three sets of microarray analysis were designed. In the first set of experiments, total RNA isolated from MCF7-ptsp53 grown at 32°C was used to generate the first-strand cDNA probe labeled with cy-5 and the cDNA probe generated from RNA of MCF7-ptsp53 grown at 37°C was labeled with cy-3. In the second set of experiments, cDNA of MCF7-ptsp53 grown at 32°C was labeled with cy-5 whereas the cDNA derived from RNA of untransfected parental MCF-7 cells grown at 37°C was labeled with

cy-3. The third set of experiment was designed to assess the effect of temperature switch on general gene expression of MCF-7 cells, as a negative control for the specific changes induced by p53 overexpression. Labeled probes were hybridized to cDNA array slides containing some 40–45K human genes. Using a criteria of 2-fold as a cutoff line, the expressions of 14 genes were shown to be differentially regulated by wt p53 overexpression in arrays that compared the wt p53 with the mutant p53 and that compared overexpressed wt p53 with the endogenous p53 but not by nonspecific temperature switch (Table 1). Interestingly, 9 out of 14 genes are functionally related to cell cycle regulation and none of the identified genes are related to apoptosis. This finding is consistent with our apoptosis assay and further supports the notion that p53 overexpression in MCF-7 cells does not induce apoptosis.

The results of array analyses revealed that four genes that function in the G₂/M phase of the cell cycle were affected by the overexpression of the wt p53. Overexpressed p53 stimulated p21 mRNA expression approximately 10-fold and suppressed transcriptions of *cdc2*, cyclin B2, and PRC1 by 50–90% of control.

We noticed that expressions of *cdc2* and cyclin B2 in the wt MCF-7 cells were slightly down regulated (40% decrease) when cells were cultured at 32°C. This may be caused by the relative slower growth rate of MCF-7 cells at 32°C as compared to 37°C. To validate the results of array studies, we checked the p21 protein levels in MCF-7, MCF7-neo, and MCF7-ptsp53 by western blot analysis. Figure 7 shows that the p21 protein level in ptsp53 cells cultured at 37°C (Lane 1) was significantly lower than neo or the wt MCF-7 cells, likely reflecting the dominant negative effect of the mutant over the wt p53 protein which is required for the basal transcriptional activity of p21 [22]. However, upon temperature shift down, the level of p21 protein was rapidly increased in MCF7-ptsp53 cells but was unchanged over the experimental duration in MCF-7 and the neo clone. It reached a maximum of 15-fold of control after 24 h in ptsp53 cells. In comparison, the temperature switch did not alter the expression of another cdk inhibitor p27. p21 has been shown to play a complementary role in the G₂/M checkpoint. Without p21 cells are unable to remain in G₂, but progress into mitosis [23]. Our results suggest that p53 induced G₂/M arrest partially through upregulation of p21. By

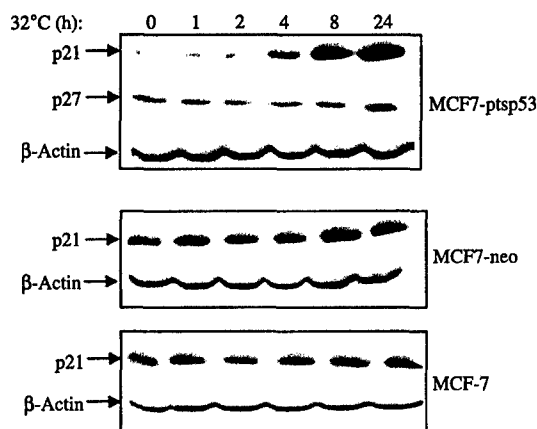


Figure 7. Induction of p21 protein expression following temperature shift down in MCF7-ptsp53 cells. MCF-7, MCF7-neo, and MCF7-ptsp53 cells were seeded at 37°C overnight. On next day, cells were switched to 32°C and incubated for the indicated hours. Western blot with anti-p21 mAb was performed to detect p21 protein expression using 50 µg of soluble proteins per cell lysate. The membranes were striped and reprobed with β-actin. The fold induction of p21 protein level normalized to β-actin relative to control (0 h point) in ptsp53 cells is as follows: 32°C 1 h, 2.7; 32°C 2 h, 2.6; 32°C 4 h, 7.2; 32°C 8 h, 13.2; 32°C 24 h, 15.6. The figure shown is representative of two separate kinetic studies. After normalization, the levels of p21 in MCF-7 and the neo clone remained constant through the experimental duration.

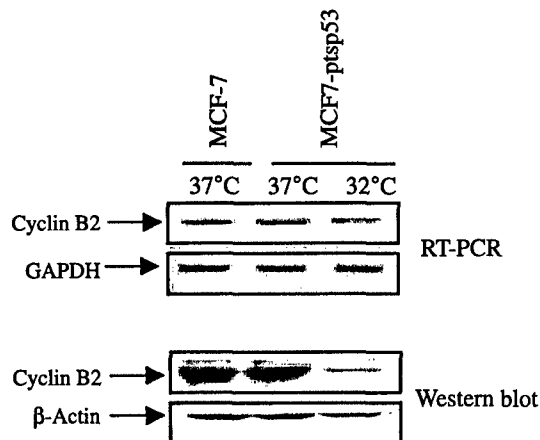


Figure 8. Cyclin B2 mRNA and protein levels decrease in ptsp53 cells upon temperature shift down. Total RNA and total cell lysate were separately harvested from MCF7-ptsp53 cells that were cultured at 37 or 32°C for 3 days. RT-PCR was conducted to detect cyclin B2 mRNA level as well as the level of GAPDH mRNA as described in Materials and methods. Compared to MCF7-ptsp53 and the untransfected MCF-7 at 37°C, cyclin B2 mRNA expressed was decreased by approximately 50% in ptsp53 cells cultured at 32°C. Detection of cyclin B2 protein was conducted by immunoblotting with 50 µg of soluble proteins per cell lysate. After normalization with β-actin, cyclin B2 protein level in ptsp53 cells at 32°C was decreased by 80% as compared to MCF-7 and ptsp53 at 37°C.

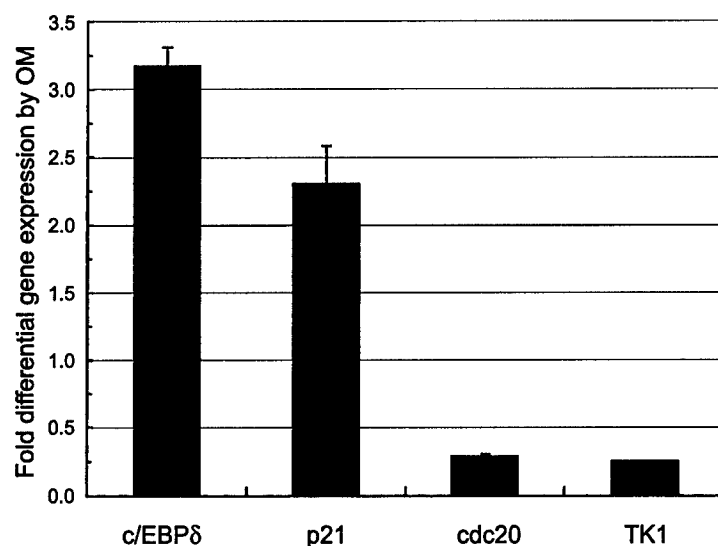


Figure 9. Microarray analysis to identify OM-regulated cell cycle components. MCF-7 cells were cultured in the absence or the presence of OM at the concentration of 50 ng/ml for 3 days and total RNAs were isolated from untreated control or OM-treated cells. The graph illustrating mRNA levels of c/EBPδ, p21, cdc20, and TK1 in OM-treated cells are presented as fold increase or fold decrease relative to control cells. The data (mean \pm s.d.) shown were derived from two independent array studies. The differential effects of OM on the expression of these four genes were also confirmed by separate microarray studies using cells treated with OM for 1 and 5 days.

using a semiquantitative RT-PCR assay and western blot, we also confirmed the repressive effect of p53 on cyclin B2, a central regulator of cell cycle progression from G₂ to mitosis (Figure 8).

OM-mediated growth inhibition is associated with a coordinate regulation of cell cycle components involved mainly in G₁/S transition

To investigate the molecular mechanisms underlying the OM-induced G₁ cell cycle arrest, microarray analysis was conducted using mRNAs isolated from untreated and OM 3-day treated MCF-7 cells. Analysis of the array data identified that the expression of four cell cycle components that function mainly in the G₁/S transition was differentially regulated by OM. Figure 9 shows that the mRNA levels of c/EBPδ and p21 were increased 3.2- and 2.3-fold by OM, respectively, whereas the mRNA levels of cdc20 and thymidine kinase 1 (TK1) were reduced to 29 and 25% of the control. Interestingly, other than p21, the expression of p53-regulated cell cycle components including cdc2, cyclin B2, and PRC1 was not altered by OM (data not shown). We further examined the effect of OM on p21 protein expression in MCF-7 cells that express functional p53 and in MCF7-ptsp53 cells

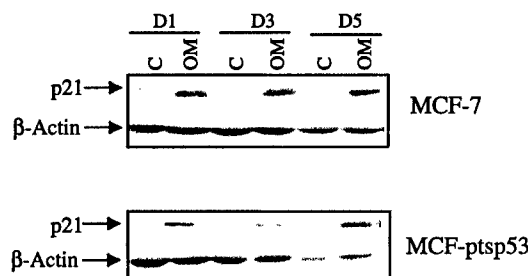


Figure 10. OM upregulates p21 protein expression in MCF-7 as well as in MCF7-ptsp53 cells. MCF-7 and MCF7-ptsp53 cells were cultured at 37°C without or with OM (50 ng/ml) for different days. At indicated time, cells were harvested for western blotting with anti-p21 mAb as described in Figure 7.

where the function of wt p53 was inhibited. Figure 10 shows that in both cell lines, the levels of p21 protein arose after exposure to OM. By 3 days of treatment, the amount of p21 protein increased more than 20-fold as compared to untreated control cells. Since the p21 mRNA only increased 2.3-fold by OM in the same period of treatment, these results suggest that the regulation of p21 by OM involves both transcriptional and translational mechanisms. Moreover, the mechanisms of regulation are independent of p53 status.

Discussion

An increase in p53 expression can result in different outcomes, including apoptosis, transient growth arrest at the G₁ and/or G₂ phase of the cell cycle, or irreversible growth arrest (senescence) [24]. The limited responses to p53 activation are cell-type specific and are largely determined by p53-down stream target genes whose transcriptions are directly or indirectly controlled by p53. In this study we demonstrate that overexpression of the wt p53 in MCF-7 breast cancer cells by culture of the ptsp53 transfectants at the permissive temperature results in a transient growth arrest at mainly the G₂/M phase of the cell cycle.

The p53-mediated G₂/M growth arrest occurred after 2–3 days of the temperature shift down. We did not observe any of the signs associated with apoptosis or senescence even when the cells were maintained at 32°C for a long period of time (up to 1 week). We sought to understand the molecular basis for this outcome in MCF-7 cells that are different from another human breast cancer cell line Hs578T where ptsp53 caused a complete G₁ arrest upon expression of the wt p53 at 32°C. Gene profiling of 40K human genes of known and unknown functions by microarray study allows us to compare the whole spectrum of changes in gene expression induced by p53. The microarray study was stratified by designing three sets of experiments that compared changes in gene expression by increased p53 expression (ptsp53 32°C v.s. MCF-7), by comparison of cells expressing functional p53 with the cells expressing the dominant negative mutant p53 (ptsp53 32°C v.s. ptsp53 37°C), and by gene changes caused by temperature switch (MCF-7 32°C v.s. 37°C). We found that in both experimental settings of p53 overexpression, wt p53 strongly induced p21 expression and suppressed cdc2, cyclin B2, and PRC1 transcription. The regulations of p53 on p21, cyclin B2, and cdc2 gene expression have been previously reported [20, 22, 23]. However, it is our novel finding that p53 down regulates PRC1.

The cyclin-dependent kinase inhibitor p21 has been demonstrated to not only play a central role in G₁/S cell cycle arrest but also to play a complementary role in the G₂/M check point [25]. Analysis of cell cycle distribution by flow cytometry showed that temperature shift down of ptsp53 cells did not increase significantly the number of cells in G₀/G₁ phase, instead it caused a marked accumulation of cells in the G₂/M phase with a concomitant decrease in the per-

centage of cells in the S phase. These data suggest that p21 contributes to the G₂/M arrest.

Cell cycle progression from G₂ to mitosis requires cyclin B and cdc2. The complex formed between cyclin B and cdc2 is essential for the G₂/M transition [26]. Repression of cyclin B1 and cdc2 transcription by wt 53 in fibroblasts resulted in a G₂/M arrest [17]. The transcription of cyclin B2 has been shown to be suppressed by wt p53 through its promoter region immediately upstream of the coding sequence [27]. Our results of array studies indicate that the transcription of cyclin B2 and cdc2 in MCF-7 cells was repressed by wt p53. In addition to cyclin B2 and cdc2, array results also revealed the down regulation of PRC1 gene. PRC1 has been shown to be involved in cytokinesis [28]. During normal cell cycle, PRC1 protein levels are high in S and G₂/M and drop dramatically after cells exit mitosis and enter G₁. Thus, it is conceivable that suppression of PRC1 transcription by wt p53 keeps cells from entering mitosis. Further study is required to determine whether PRC1 is a new target gene of p53. Interestingly, analysis of array results from both sets of experiments did not find p53-induced changes of genes involving in apoptosis such as bax. This corroborates our observation for the lack of apoptotic features in MCF7-ptsp53 cells when cultured at 32°C.

One of the aims of this study was to investigate whether p53 is directly involved in the OM-induced growth inhibition of MCF-7 cells. We postulated that a decreased p53 expression might persuade cells to undergo growth arrest instead of apoptosis. However, by using the MCF7-ptsp53 cells we found out that inhibiting the transcriptional function of endogenous p53 by expression of the mutant p53 at 37°C did not enhance or prevent the antiproliferative activity of OM and did not trigger an apoptotic response to OM. Our results suggest that p53 is not directly involved in the process of OM-induced growth repression and morphological changes. Treatment of cells with OM leads to profound changes in the expression of many genes. Therefore, even though OM induced growth repression and p53 down regulation occur concomitantly, it is not totally surprising that these two biological effects are not directly connected. Microarray analysis indicates that OM affects the cell growth through regulation of genes that mainly function in the G₁/S transition, including c/EBP δ , cdc20, TK1, and p21.

Overall, our studies of cell cycle and microarray analyses suggest that OM and p53 arrest cell growth by affecting different phases of the cell cycle; OM

arrests cells at the G₀/G₁ phase whereas p53 halts the cycle progression at the G₂/M phase. In addition to regulate the cell growth, OM has been shown to induce many changes in cellular functions such as cell morphology, adhesion, motility, metabolism, and extracellular matrix deposition. Similarly, p53 controls numerous critical cellular processes. It has been shown that changes in p53 levels lead to variable outcomes. Given the consideration of the importance of p53 in cell homeostasis, the regulation of OM on p53 transcription warrants further investigation.

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References

- Zarling JM, Shoyab M, Marquardt H, Hanson MB, Lionbin MN, Todaro GJ: Oncostatin M: a growth regulator produced by differentiated lymphoma cells. *Proc Natl Acad Sci USA* 83: 9739-9743, 1986
- Liu J, Spence MJ, Wallace PM, Forcier K, Hellstrom I, Vestal RE: Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth and down-regulation of the c-myc proto-oncogene. *Cell Growth Diff* 8: 667-676, 1997
- Spence MJ, Vestal RE, Liu J: Oncostatin M-mediated transcriptional suppression of the c-myc gene in breast cancer cells. *Cancer Res* 57: 2223-2228, 1997
- Douglas AM, Grant SL, Goss GA, Clouston DR, Sutherland RL, Begley CG: Oncostatin M induces the differentiation of breast cancer cells. *Int J Cancer* 75: 64-73, 1998
- Harris CC: Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst* 88: 1442-1455, 1996
- Liu J, Ahlborn TE, Li C, Spence MJ, Boxer LM: Transcription of tumor suppressor p53 gene is down-regulated in differentiated breast cancer cells induced by cytokine oncostatin M and PMA. *Cell Growth Diff* 10: 677-683, 1999
- Li C, Ahlborn TE, Tokita K, Boxer L, Noda A, Liu J: The critical role of the PE21 element in oncostatin M-mediated transcriptional repression of the p53 tumor suppressor gene in breast cancer cells. *Oncogene* 20: 8193-8202, 2001
- Michalovitz D, Halevy O, Oren M: Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant p53. *Cell* 62: 671-680, 1990
- Milner J, Medcalf E: Temperature-dependent switching between "wild-type" and "mutant" forms of p53Val135. *J Mol Biol* 210: 481-484, 1990
- Martinez J, Georgoff I, Levine AJ: Cellular location and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev* 5: 151-159, 1991
- Hughes A, Gollapudi L, Sladek T, Neet K: Mediation of nerve growth factor-driven cell cycle arrest in PC12 cells by p53. *J Biol Chem* 275: 37829-37837, 2000
- Liu X, Nishitani J, McQuirter JL, Baluda MA, Park NH: The temperature sensitive mutant p53-143ala extends *in vitro* life span, promotes errors in DNA replication and impairs DNA repair in normal human oral keratinocytes. *Cell Mol Biol* 47: 1169-1178, 2001
- Eliyahu D, Evans S, Rosen N, Eliyahu S, Zwiebel J, Paik S, Lippman M: p53Val135 temperature sensitive mutant suppresses growth of human breast cancer cells. *Breast Cancer Res Treatment* 30: 167-177, 1994
- Pollock R, Lang A, Ge T, Sun D, Tan M, Yu D: Wild-type p53 and a p53 temperature-sensitive mutant suppress human soft tissue sarcoma by enhancing cell cycle control. *Clinical Cancer Res* 4: 1985-1994, 1998
- Willers H, McCarthy E, Wu B, Wunsch H, Tang W, Taghian D, Xia F, Powell SN: Dissociation of p53-mediated suppression of homologous recombination from G₁/S cell cycle checkpoint control. *Oncogene* 19: 632-639, 2000
- Agarwal M, Agarwal A, Taylor W, Stark GR: p53 controls both the G₂/M and the G₁ cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 92: 8493-8497, 1995
- Taylor W, DePrimo S, Agarwal A, Agarwal M, SchÖnthall A, Katula K, Stark G: Mechanisms of G₂ arrest in response to overexpression of p53. *Mol Biol Cell* 10: 3607-3622, 1999
- Wang F: Analysis of downstream effectors of p53 on cell growth arrest and apoptosis induced by a temperature-sensitive Val138 mutant. *J Med Dent Sci* 45: 141-149, 1998
- Chylicki K, Ehinger M, Svedberg H, Gullberg U: Characterization of the molecular mechanisms for p53-mediated differentiation. *Cell Growth Diff* 11: 561-571, 2000
- Sugrue MM, Shin DY, Lee SW, Aaronson SA: Wildtype p53 triggers a rapid senescence program in human tumor cells lacking functional p53. *Proc Natl Acad Sci USA* 94: 9648-9653, 1997
- Dubrez L, Coll J, Hurbin A, Fraipont F, Lantejoul S, Favrot M: Cell cycle arrest is sufficient for p53-mediated tumor regression. *Gene Therapy* 8: 1705-1712, 2001
- Tang H, Zhao K, Pizzolato JF, Fonarev M, Langer JC, Manfredi JJ: Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. *J Biol Chem* 273: 29156-29163, 1998
- Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown J, Sedivy J, Kinzler K, Vogelstein B: Requirement for p53 and p21 to sustain G₂ arrest after DNA damage. *Science* 282: 1497-1501, 1998
- Bargonetti J, Manfredi J: Multiple roles of the tumor suppressor p53. *Curr Opin Oncol* 14: 86-91, 2002
- Chan TA, Hwang PM, Hermeking H, Kinzler KW, Vogelstein B: Cooperative effects of genes controlling the G(2)/M checkpoint. *Genes Dev* 14: 1584-1588, 2000

26. Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T, Maller JL: Cyclin is a component of maturation-promoting factor from *Xenopus*. *Cell* 60: 487-494, 1990
27. Kruse K, Wasner M, Reinhard W, Ulrike H, Dohna C, Mössner J, Engeland K: The tumor suppressor protein p53 can repress transcription of cyclin B. *Nucl Acids Res* 28: 4410-4418, 2000
28. Jiang W, Jimenez G, Wells N, Hope T, Wahl G, Hunter T, Fukunaga R: PRC1: a human mitotic spindle-associated CDK substrate protein required for cytokinesis. *Mol Cell* 2: 877-885, 1998
29. Liu J, Hadjokas N, Mosley B, Estrov Z, Spence MJ, Vestal RE: Oncostatin M-specific receptor expression and function in regulating cell proliferation of normal and malignant mammary epithelial cells. *Cytokine* 10: 295-302, 1998

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